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EFFECT(S) OF PHARMACOLOGIC INTERVENTION ON OXYGENATION, LUNG
WATER AND PROTEIN LEAK IN THE THE PSEUDOMONAS ARDS PORCINE
MODEL

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SUMMARY

The adult respiratory distress syndrome is a condition which occurs as a result of both direct and indirect pulmonary injury. The mortality rate of the syndrome, which may effect previously fit patients, is over 50% and higher where sepsis predominates. This mortality, despite modern techniques in intensive care, has hardly changed in 20 years. The pathophysiological changes in the condition result in respiratory failure requiring endotracheal intubation and mechanical ventilation, the appearance of diffuse fluffy infiltrates on chest roentgenogram, a normal pulmonary wedge pressure, an arterial:alveolar pO₂ ratio ≤ 0.2 , and a total static lung compliance of ≤ 50 ml H₂O.

At the cellular level, the lung injury is due to damage of the alveolar-capillary membrane by various mediators produced from circulating elements of the blood, most notably neutrophils. Neutrophils become "activated" by contact with a soluble or phagocytic stimulus, then break down and release inflammatory mediators. Among the mediators produced, following a septic stimulus are the products of the cyclooxygenase and lipoxygenase systems of arachidonic acid metabolism such as the prostaglandins, leukotrienes, the complement factors C5a, C3a which are also mobilized by septic stimuli. Oxygen free radicals and tumor necrosis factor (TNF) are other inflammatory mediators released by host cells. TNF may cause host tissue damage by activating neutrophils, but there is also evidence that it may have a direct deleterious effect on the alveolar-capillary membrane.

All of these substances can be toxic to the alveolar capillary membrane and eventually may cause its disintegration with concomitant protein leak across the damaged membrane into the lung. There is however, a growing body of opinion that neutrophil adhesion to the

endothelium is necessary for neutrophil induced endothelial damage.

When the lymphatic clearance capacity of the lung is exceeded, pulmonary edema occurs and the clinical picture seen in ARDS unfolds. Pseudomonas-induced ARDS in the porcine model has been used as an effective and reproducible model of sepsis-induced ARDS in this laboratory.

Because ARDS is mediated by numerous inflammatory mediators, it is likely that treatment will require several pharmacological blocking agents. We have previously established that treatment with cimetidine, or ranitidine in combination with diphenhydramine, H₂ and H₁ blockers, respectively, and ibuprofen, a prostaglandin antagonist (CID), given i.v. at 20 and 120 minutes after pseudomonas infusion, significantly attenuates both the early hypertensive and late permeability phases of the syndrome as measured by hemodynamic parameters, blood gases, bronchoalveolar lavage protein content and extravascular lung water.

We have established that platelet-activating factor is present in pig lung after sepsis. An anti-platelet activating factor, SRI 63-675, has been shown previously to attenuate the early phase of pulmonary hypertension and possibly improve the late permeability changes in the model but caused severe hemolysis, thus making it impractical for clinical use. Experiments using superoxide radical scavengers in the model have not been shown to be effective in improving the response to injury in any way despite the wealth of evidence that primary oxygen radicals effect alveolar-capillary membrane damage. However the characterization of superoxide production from neutrophils in this model has been much more clearly defined in this laboratory in the last year. Similarly the production of a group

of longer lived but more potent oxidants, hypochlorous acid and monochloramines, has been identified and quantified from onset to advanced sepsis. Treatment with the cyclooxygenase blocker, ibuprofen, markedly attenuates the release of a wide variety of neutrophil oxidants. These data indicate that cyclooxygenase products of arachidonic acid are intimately involved in the respiratory burst of phagocytes.

Neutrophil counts taken throughout the five hour study period show the development of a profound peripheral neutropenia (Fig 1). This coincides with the accumulation of neutrophils in the lung and the development of the lung injury. Measurement of neutrophil adhesion receptor expression shows a significant increase in septic animals compared with controls. Not only does this coincide with the neutropenia, but the expression of adhesion receptors on neutrophils isolated from the alveoli of septic animals is greater than that seen in the blood. Both these factors implicate altered adhesion receptor expression in the development of the neutropenia and the sequestration of neutrophils in the lung.

As mentioned in a previous report, kinetic and quantitative studies of superoxide production from neutrophils separated from the pre- and post-injury phases in the model indicate that *Pseudomonas* primes these cells to produce superoxide anion at a much higher rate post-injury than pre-injury, thus implicating neutrophil generated superoxide anion in the endothelial cell damage. Alveolar macrophages retrieved from bronchoalveolar lavage in the post-injured phase are capable of generating large quantities of superoxide anion and hydrogen peroxide and may have a role in the injury process, in particular with epithelial cell damage.

Tumor necrosis factor (TNF) generated by mononuclear cells is increased in the

injured animal. Following the bacterial insult there is an immediate and dramatic rise in TNF levels. This substance has been implicated in many inflammatory processes and has been seen by others to increase in humans exposed to endotoxin. Raised levels of TNF (measured in plasma using the L929 cytotoxicity assay) are temporally related to alterations in hemodynamic parameters in this model system.

Specialized phagocytic cells which comprise the reticuloendothelial system (RES) clear circulating particles (i.e. bacteria, cell debris, endotoxin, apoptotic cells) from blood. Conventionally, the RES is considered to be localized to bone marrow, liver and spleen. Mononuclear phagocytes (i.e., macrophages) constitute the RES and reside in and out of the vascular spaces of these organs. Recently, cellular constituents of the mononuclear phagocyte system have been identified within the microcirculation of the lung. Pulmonary intravascular macrophages (PIMs), distinct from alveolar and interstitial macrophages, originate from blood monocytes and reside in the lung adhered to luminal surfaces of the thick portion of interalveolar capillaries. PIMs are anchored to the microvascular endothelium by intercellular adhesion plaques. Recent evidence suggests that PIMs are present in human lung as well.

FOREWORD

In conducting the research described in this report, the investigators adhere to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 78-23, Rev. 1978).

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PROBLEM

The adult respiratory distress syndrome (ARDS), as first described by Ashbaugh (1) 20 years ago, is a pathophysiological pulmonary condition of multiple etiologies. The syndrome may be initiated by direct pulmonary injury or may be seen as the lungs' response to a remote or systemic insult. In civilian life, the most common causes of ARDS are multiple trauma, aspiration of gastric contents, sepsis and pancreatitis. In combat soldiers, the condition known as the traumatic wet lung syndrome during the Korean conflict and Da-Nang lung in the Viet Nam War, is now recognized as ARDS and results from blast injuries, direct lung contusion, burn inhalation, inhalation of toxic substances, aspiration, multiple transfusions and as a complication of sepsis. As such the condition often affects previously fit and healthy patients with a considerable mortality.

Clinically, patients are considered to have the syndrome when certain criteria are met: respiratory failure requiring endotracheal intubation and mechanical ventilation, the appearance of diffuse pulmonary infiltrates on chest roentgenogram, an initial pulmonary wedge pressure of 18 mmHg or less, an arterial to alveolar P_{O_2} ratio ≤ 0.2 and a total static lung compliance of ≤ 50 ml H_2O which increases the work of respiration with stiffer lungs and decreases oxygenation.

Applying these criteria, between 150,000 and 200,000 patients in the United States are affected annually by this syndrome. The mortality rate remains at 50% or higher where sepsis predominates (2). The mainstay of treatment is supportive therapy, treatment of the underlying disorder, the maintenance of adequate oxygenation with mechanical ventilation and positive endexpiratory pressure (PEEP), fluid balance, nutrition and antibiotics where

indicated, since patients with ARDS are more susceptible to nosocomial infections.

BACKGROUND

Despite the multiple causes of ARDS, the final pathways end in the same result, i.e., damage to the alveolar-capillary membrane with increased permeability and accumulation of water and protein rich fluid in the pulmonary interstitium. Alveolar flooding occurs when interstitial and lymphatic clearance capacity are exceeded leading to decreased pulmonary compliance. The perfusion of unventilated alveoli manifested as hypoxemia is also a feature. This hypoxemia is refractory to increased inspired oxygen concentrations.

It is likely that successful treatment of ARDS will involve intervention to prevent capillary endothelial damage and protein leak across the membrane, as well as methods to increase compliance and improve oxygenation.

Measurement of extravascular lung water by the indicator dilution technique has been used in all the animal experiments in this laboratory where such determinations were necessary for the assessment of therapeutic intervention in experimental ARDS. In addition to these methods of determining lung leak, we have added the technique of bronchoalveolar lavage to further study cell traffic across the damaged alveolar-capillary membrane and to recover inflammatory and resident cells from the alveolus in order to study their role in the development of the syndrome.

In addition to the comprehensive evaluation of clinical parameters in this model, we have also focused on a wide array of cellular, molecular and functional assessments of abnormalities in sepsis-induced ARDS. These new parameters include assay of short and long lived oxidant generation from neutrophils and tumor necrosis factor and interleukin-1 generation from mononuclear cells. Myeloperoxidase production and gene expression in

alveolar macrophages and circulating neutrophils are a new focus and neutrophil adherence receptor (CD18) studies utilising flow cytometry and a porcine pulmonary endothelial cultured cell line are another of our current interests. Assay of intracellular protein kinase C activity in phagocytes and investigation into the role of the recently described cell line, the pulmonary intravascular macrophage (PIM) also provide exciting new directions.

Functional properties of these cells have been investigated in-vivo. Several studies have demonstrated that PIMs phagocytose cellular (erythrocytes, bacteria) and noncellular (endotoxin) matter. Warner et al used histologic techniques in sheep to show that infused iron oxide particles localized in lung vascular phagocytes which satisfied histochemical criteria for cells of monocyte/macrophage lineage. Bertram et al harvested PIMs from porcine lung and showed that isolated cells formed both lipooxygenase and cyclooxygenase metabolites of arachidonic acid when exposed to calcium ionophore in-vitro. Thus far, however, limited additional functions have been identified. Staub reported that mononuclear phagocytes obtained from goat lung produced enhanced quantities of superoxide anion and tumor necrosis factor following protein kinase C activation by phorbol myristate acetate. Establishing additional fundamental "macrophage-like" properties of pulmonary intravascular macrophages (i.e., antigen presentation, growth factor secretion, cytolytic function) is needed.

Techniques which permit harvest of PIMs from the lungs for ex-vivo study have been described. Bertram and Morton obtained porcine PIMs by removing the heart and lungs en-bloc with subsequent vascular perfusion of the lung ex-vivo with collagenase. This technique is limited by the inherent time delays resulting from extensive dissection. Further, cell yields reported may not be optimal if large cell numbers are required. Staub reported slightly

higher cell yields by ex-vivo perfusion of lung using chelating agents (e.g., EDTA) as opposed to enzymatic removal.

In this report we describe an in-situ technique for isolation of anchored mononuclear phagocytes from the pulmonary microcirculation of young swine. This is an original technique which has been developed in our laboratory over the last 12 months. The technique reported requires limited dissection and permits rapid retrieval of anchored mononuclear phagocytes in large numbers from post mortem lung. Use of the technique should facilitate experimentation and minimize potential artifact produced by delays in procurement.

RATIONALE

ROLE OF INFLAMMATORY MEDIATORS IN ARDS.

It is clear that the lung injury in ARDS is mediated by a large number of substances. That some of these substances are inter-related and share a common final pathway or common enzyme system is becoming more obvious. What is not clear is the exact inter-relations between these mediators.

Central to the lungs' response to injury in sepsis induced ARDS is the neutrophil. Sequestration of neutrophils takes place soon after endotoxin infusion in-vivo. The exact method of neutrophil aggregation is not known, but it is hypothesized that substances such as complement (C3a and C5a), leukotrienes and various other chemotactic substances are involved in the initiation of the process, and that this process involves an increased adhesiveness between neutrophils and between neutrophils and endothelium. This increased adhesiveness is mediated by altered expression of glycoprotein adhesion receptors on the surface of neutrophils and endothelial cells alike. In vitro studies have shown that exposing neutrophils and endothelium to inflammatory mediators increases the expression of these receptors and that this coincides with increased adhesion. Further in vitro studies blocking the adhesion receptors on the surface of neutrophils, using monoclonal antibodies which bind to functional epitopes on the receptor, cause a marked decrease in the adhesion of stimulated neutrophils.

Neutrophils become "activated" when they come in contact with a soluble or phagocytic stimulus and manifest this activation as an increased respiratory burst with an increase in oxygen consumption, activation of the hexose monophosphate shunt and

generation of reactive oxygen species and their metabolic products. These products may be injurious to endothelial cells as well as deactivating enzymes. It is felt that the combination of reactive oxygen species, proteases and the protected microenvironment afforded by the adhesion of neutrophils to endothelium is especially important as the host's anti oxidant and anti-protease mechanisms are rendered ineffective by a process of exclusion from the adherent cell membranes. In this way adhesion of phagocytes to endothelium actually facilitates cell damage.

In addition to oxygen free radicals, the neutrophils, platelets, monocytes and lymphocytes can release a number of other factors which have an affect on pulmonary hemodynamics as well as act directly on the endothelium. Arachadonic acid metabolites produced by the circulating elements in the blood are thought to cause the acute pulmonary hypertension seen immediately after endotoxin infusion as well as an increased lung lymph flow. These effects are thought to be caused by products of the cyclooxygenase system of arachadonic acid metabolism, since increased plasma levels of TxB₂ and 6 Keto PGF₁α are temporally related to the initial pulmonary hypertension and increased lung lymph flow. It has been shown by previous experiments in this laboratory that these effects can be prevented by a combination of an anti-prostaglandin in conjunction with histamine receptor blockers. The macrophage has been implicated in the lung injury by its release of substances such as the interleukins and tumor necrosis factor (cachectin).

The second phase of the lungs' response to endotoxin is characterized by a sustained but lower increase in pulmonary artery pressure and a protein rich lymph flow secondary to an increased capillary permeability. This late phase of pulmonary hypertension and protein

leak is not thought to be due to the metabolites from the arachadonic cascade; however, it is possible that oxygen-free radicals generated by neutrophils are responsible for a large part of the vascular endothelial cell damage as well as proteolytic enzymes released by degranulating neutrophils all of which result in increased permeability.

Oxygen-free radicals produced by neutrophils are normally converted to non-injurous substances by the enzymes superoxide dismutase and catalase. However, when these mechanisms are overcome by overwhelming oxygen radical production (O_2^- , H_2O_2 , OH^\cdot), or when these mechanisms are excluded from the microenvironment, endothelial cell damage can occur. The reaction of hydrogen peroxide with chloride ions in the presence of the neutrophil degranulation enzyme, myeloperoxidase, leads to the production of hypochlorous acid which is thought by some investigators to be the most potent of the oxidants produced by neutrophils. Hypochlorous acid can react with a number of intermediate compounds forming a series of longer lived and more stable oxidant compounds. This array of oxidants is a formidable insult to both invading pathogens and host cells. The hydroxyl radical particularly causes peroxidation of lipid membranes and, in the presence of transition metals such as copper and iron, these membranes disintegrate, lose their integrity and result in increased permeability.

EXPERIMENTAL METHODS AND STUDIES OUTLINED.

THE MODEL

The porcine model was used in all experiments. Young swine weighing between 15-25 kgs were anesthetized with intramuscular ketamine hydrochloride 25 mg/kg and placed supine. Anesthesia was induced with sodium pentobarbital (10 mg/kg) and maintained with intermittent bolus pentobarbital as necessary. Following intubation with a cuffed endotracheal tube; they were paralyzed with continuous intravenous pancuronium bromide (0.2 mg/min) to permit mechanical ventilation with 0.5 FiO₂, 5 cm H₂O positive end expiratory pressure (PEEP) and 20cc/kg tidal volume at a rate which produced a PaCO₂ of approximately 40 torr at the beginning of the experiment.

A less lethal model than that used previously is at present being employed. Live *Pseudomonas aeruginosa* (PAO strain, 5×10^8 CFU/ml at 0.3 ml/20kg/min) was administered for 1 hour by continuous infusion, rather than for the entire length of the study. This has been shown to produce a marked physiological deterioration, representative of acute ARDS, resulting in an immediate significant increase in pulmonary artery pressure (PAP) which persists throughout the entire duration of the experiment. Systemic arterial pressure (SAP) shows a progressive decline as does cardiac index (CI) and PaO₂. Extravascular lung water (EVLW) becomes significantly elevated when compared to saline controls.

Catheters were inserted into the left common carotid artery for monitoring SAP and arterial blood gases, and into the right and left external jugular veins for infusion of *Pseudomonas* (Ps) and the therapeutic agents to be studied. A thermodilution Swan-Ganz catheter was passed through the right jugular vein into the pulmonary capillary and wedged

in position with the balloon inflated. This was used to monitor PAP, pulmonary wedge pressure (PWP), and thermodilution cardiac output. Cardiac output (CO) was converted to cardiac index (CI) by the formula:

$$CI = CO / 0.112 BW^{2/3}$$

where BW is the body weight in kg. Blood gases were measured with a blood gas analyzer (Instrumentation Laboratories, Model 113).

A 5 French femoral artery lung water catheter (American Edwards Laboratories, Model 96-020-5F) was passed into the lower abdominal aorta for measurement of thermal cardiogreen extravascular lung water (EVLW). In this technique 10 ml of iced, green dye solution (2 mg indocyanine green dye in 10 ml 5% dextrose) were injected as a bolus through the proximal port of the Swan-Ganz catheter as blood was simultaneously withdrawn through the thermistor-tipped femoral artery catheter and a densitometer cuvette (Waters Instruments Inc., Model 402A) linked to a lung water computer (American Edwards Laboratories, Model 9310). The computer measured the mean transit times of the intravascular dye (MTD) and freely diffusible thermal component (MIT) as well as the cardiac output (CO). EVLW was calculated by the formula:

$$EVLW = \frac{CO (MTD-MIT)}{BW (kg)}$$

Bronchoalveolar lavage was performed at 0 minutes and at 5 hours to harvest resident alveolar macrophages and PMNs and to provide alveolar lining fluid for biochemical studies including protein content. At the five hour time point the migrated alveolar

neutrophils were isolated and examined by direct immunofluorescent flow cytometry to quantitate CD18 adhesion receptor expression.

Peripheral blood samples were withdrawn at baseline (zero timepoint), 1 hour (i.e. directly after the *Pseudomonas* infusion) and at 5 hours (end-stage sepsis) and neutrophils were isolated using dextran sedimentation and Ficoll-sodium diatrizoate density gradient centrifugation. Assay of phorbol ester stimulated production of oxygen dependant neutrophil products were performed as outlined below. The volume of blood withdrawn was determined by the peripheral white blood cell count measured 15 minutes prior to the designated time points. Similarly arterial blood samples were withdrawn at 15 minute intervals for estimation of tumor necrosis factor levels and measurement of CD18 receptor expression on peripheral neutrophils.

NEUTROPHIL OXIDANT GENERATION STUDIES.

Many investigators have established that human and various animal species produce toxic oxygen metabolites from neutrophils in vitro. Neutrophils become activated by contact with a soluble or particulate stimulus such as live bacteria, endotoxin (lipopolysaccharide) and undergo a respiratory burst with subsequent production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). We performed studies to document and properly characterize the kinetics of O_2^- release in addition to the total amount of these metabolites released from pseudomonas-primed porcine neutrophils. Neutrophils are separated from the pre- and post-injury blood samples and adjusted to a known concentration of cells per milliliter. For kinetic studies pre-injury and post-injury neutrophils are stimulated with phorbol myristate acetate (PMA) in a dual beam spectrophotometer in the presence of

cytochrome C at 550 nm. The rate of superoxide dismutase inhibitable O_2^- production per minute is directly proportional to the rate of cytochrome C reduction.

In order to determine the direct stimulatory effect of live organisms upon neutrophil free radical generation without opsonization by serum factors and to eliminate the effect of endogenously produced inflammatory mediators upon reactivated oxygen intermediate production, a series of in-vitro studies were performed. In these studies, arterial blood (25ml) obtained from healthy young swine ($n=5$) was mixed in plastic syringes with EDTA (0.1 ml, 15% EDTA/10 mls whole blood) at 0 min and 60 min following the 60 min infusion of 0.9% NaCl and neutrophil isolation performed as above. Superoxide anion production following PMA stimulation was assessed immediately following isolation of neutrophils from both samples. Neutrophils were then admixed in a ratio of 1:100 with live bacteria and incubated for 60 minutes at 37° . Controls for these studies contained no bacteria but the identical number of neutrophils incubated under the same conditions. At the end of the 60 minute incubation, cytochrome C and PMA were added to reaction mixtures and the change in optical density was followed continuously over a ten minute period as for the ex-vivo PMNs studies described above. The nanomolar extinction coefficient of 0.0211 was used to quantify cytochrome C reduction. The production of superoxide anion was calculated for each minute and plotted against time. Rates are expressed as nmols of O_2^- /min/ 10^6 PMNs.

IBUPROFEN STUDIES.

To date this non steroidal anti-inflammatory drug has been proven to be the most effective drug in ameliorating the derangement in hemodynamic parameters in ARDS. Its

effects have been shown to be due to the blockade of cyclooxygenase metabolites of arachidonic acid, in particular thromboxane. There are data from in-vitro studies to suggest that ibuprofen may have an effect on neutrophil oxidant generation by altering the intracellular NADPH-oxidase system. We have previously shown that pretreatment of animals with ibuprofen caused a significant attenuation of the respiratory burst. We now progressed to look at the effects of post treatment with this drug on the production of neutrophil oxidants in both treated and untreated animals, as this would have far greater practical application in the field.

LONG-LIVED OXIDANT ESTIMATION.

Current evidence suggests that a system composed of H_2O_2 , the azurophilic granular enzyme myeloperoxidase (MPO) and a halide (H_2O_2 -MPO-Halide) constitute a source of another group of potent toxic oxidants produced from neutrophils. The hypohalous acids (HOCL, HOBR, HOI) and monochloramines are compounds active in the intracellular killing of micro-organisms within phagocytic vacuoles. These compounds are also secreted by stimulated PMNs and may attack local targets or modify neutrophil proteolytic enzyme behaviour. In fact, it is widely believed that this is the most potent oxidant system associated with neutrophil cytotoxic function. The role of the long lived oxidants is not well defined. However, the biological reactivity and cytotoxic potential of the powerful oxidant, hypochlorous acid, and its chloramine derivatives (e.g. taurine chloramine) suggest that these active oxidants play an important role in the inflammatory response and in host defense. Moreover, mounting evidence implicates products of this system not only as direct cytotoxic agents but as potent modulators of the neutrophil inflammatory response. The known actions

of the long lived oxidants not only include antibacterial activity but also inactivation of alpha-1-proteinase inhibitor allowing unrestricted elastase mediated tissue damage (particularly in the lung), as well as activation of the latent proteinase, collagenase, generating a full proteolytic enzyme which specifically destroys collagen. Finally, they permit oxidation of chemical compounds such as drugs, industrial pollutants and environmental chemicals to damaging electrophilic free radical forms. The importance of this system is that it allows neutrophils to convert the short lived non-specific effects of primary oxygen radicals into highly specific long-acting effects by producing compounds which modify cellular or plasma constituents critical to the inflammatory response.

This laboratory has recently used a continuous spectrophotometric assay which can determine the amount and kinetics of hypohalous acid production by phagocytic cells.

The assay employs the ability of the amino acid, taurine, to act as a scavenger of the hypohalous acids, the resulting taurine halides can oxidize 5-thio-2-nitrobenzoic acid (TNB) to the disulfide, 5-5'-dithiobis (2-nitrobenzoic acid DTNB); the change in optical density measured at 412nm allows estimation of the production of hypohalous acids.

ENDOTHELIAL CELL LINE AND FLOW CYTOMETRY STUDIES.

It is unclear whether activation of neutrophils by prior exposure to phagocytic stimuli also alters the adherence and cytotoxic potential of these cells. By utilizing a cultured endothelial cell line it is possible to determine the ability of artificially stimulated neutrophils to adhere to, or to cause cell lysis of, cultured endothelial cells (pulmonary artery endothelium or, preferably, pulmonary microvascular cells). In conjunction with this, we are studying the

regulation of neutrophil adherence glycoproteins by flow cytometry. It is generally agreed that a receptor interaction is the means by which neutrophils adhere to the vascular endothelium, and most investigators believe that the most important receptor is the CD-18 glycoprotein. We are interested in studying the physiology of this receptor during sepsis in the porcine model. We hope to be able to block the receptor using a monoclonal antibody, 60.3, which recognises the CD18 antigen complex. This monoclonal antibody is also being used as an immunofluorescent label to allow us to monitor the upregulation of the CD18 receptor during sepsis and during different treatment modalities.

To perform direct immunofluorescent staining, the monoclonal antibody must first be conjugated with a fluorescent tag. Monoclonal antibody 60.3 and a non specific isotypic control were both conjugated with fluorescein isothiocyanate (FITC). Monoclonal antibodies were purified by ammonium sulphate fractionation and dialyzed against a carbonate \ bicarbonate buffer.

This obtains an optimal pH for conjugation and the dialysis removes any inhibitors of conjugation such as amines or azide. After incubating the antibodies with the FITC the excess fluorochrome is removed on a desalting column. The conjugated monoclonal antibody is now examined and the fluorochrome:protein ratio is calculated spectrophotometrically, using the formula

$$\text{Optical Density (OD)} = 2.87 \times \text{OD}_{495} / \text{OD}_{280} - [0.35 \times \text{OD}_{495}]$$

The fluorescenated monoclonal antibody is then titrated to determine antibody excess and the antibody frozen at -70° C in appropriately sized aliquots.

During each experiment blood samples are drawn into polypropylene tubes containing

EDTA and sodium azide. The specimens are immediately placed on ice and kept at 4°C throughout the experiment. Blood from each time point is incubated with both monoclonal 60.3 or the isotypic control for 20 min at 4°C using antibody excess, as previously determined. Specimens are then washed twice with PBS containing 0.1% bovine serum albumin, 0.1% EDTA and 0.1% sodium azide. Following this the red cells are lysed using an ammonium chloride lysis buffer. The blood leukocytes are suspended in 1% paraformaldehyde to fix the cells. Fixed cells are examined by flow cytometry. A Becton Dickinson machine with a four decade log amplifier is used. Blood neutrophils are gated according to their light scatter properties, and the mean channel fluorescence of the gated cells is calculated. To permit quantification, commercially available fluorescent microbead standards were employed to convert mean channel fluorescence, which is expressed on a logarithmic scale, to equivalent soluble fluorescent units on a linear scale. Subsequent division by the fluorochrome : protein ratio gives the actual number of antibodies on the cell surface. This procedure is also performed on neutrophils harvested from the airspace by bronchoalveolar lavage.

TUMOR NECROSIS FACTOR (TNF) STUDIES.

The L929 mouse fibroblast cell assay, as described by Fick and Gifford, was used to measure plasma TNF activity. L929 cells were seeded into flat-bottom 96 well microtiter plates (Corning, NY) at a density of 4×10^4 cells/well and grown to confluence overnight in Dulbecco's minimal essential medium (GIBCO) containing 1% penicillin-streptomycin and 5% fetal calf serum (DMEM). Medium was then removed from confluent monolayers and

100 ul of fresh DMEM containing Actinomycin-D (Merck Sharp & Dohme, Weston, PA) (final concentration 5ug/ml) was added to each well. One hundred ul of each of the following was added to selected duplicate wells containing L929 cells: 1) DMEM over L929 monolayers (0% cytotoxicity); 2) serial dilutions of recombinant TNF α (5×10^3 to 6×10^4 U/ml (Cetus Corp., Emeryville, CA); 3) plasma samples from control or septic pigs; 4) DMEM in blank wells without cells (100% cytotoxicity). Plates were then incubated for 20 hours (37°C, 5% CO₂). Following incubation, the medium was removed and adherent L929 cells were stained for 10 mins with 0.5% crystal violet in 20% methanol, rinsed in water and air dried.

Optical density of each well was determined by a microplate reader (Bio-Tek EL 309) and calibrated to non-cellular reagent blanks at a wavelength of 550 nm. The % cytotoxicity of L929 cells was determined by the formula

$$\% \text{ cytotoxicity} = \frac{\text{OD wells with 0\% cytotoxicity} - \text{OD experimental sample wells}}{\text{OD wells with 0\% cytotoxicity}}$$

In studies where TNF activity is presented in units/ml, one unit of TNF activity is defined as that equal to 50% L929 cytotoxicity.

To determine whether cytolytic activity in plasma was due to TNF α or TNF β , 120 min prior to L929 assay, 100ul of the septic or non-septic plasma to be tested was incubated separately with 100ul of 60 neutralizing units/ml (NU/ml) of polyclonal anti-human TNF α (Endogen

Inc., Boston) , or 200 NU/ml of polyclonal anti-human TNF β (Endogen Inc., Boston), or with combined antibodies. Aliquots of the same plasma samples were also incubated without TNF antibodies to determine % cytotoxicity without antibody interference in these samples. All of these plasma samples with and without antibodies were then tested for TNF activity. Percent L929 cytotoxicity was determined as outlined above and the results compared.

PULMONARY INTRAVASCULAR MACROPHAGE STUDIES

Yorkshire pigs (13-25 kg) were obtained from a commercial vendor in the Richmond area and housed in the Virginia Commonwealth University Vivarium for 6-8 days prior to use. All animals received prophylactic administration of benzethine and procaine penicillin (300,000 Units each, intramuscular) upon arrival. This antibiotic regimen lowered the frequency of respiratory tract infections previously observed in animals housed in the close quarters of the animal care facility. No animal was entered into the study within 48 hours of receiving antibiotics.

Animals were pre-anesthetized with I.M. ketamine (25 mg/kg) and restrained supine. General anesthesia was induced by intravenous pentobarbital (30 mg/kg). Tracheal intubation was performed using a cuffed endotracheal tube (National Catheter) and mechanical ventilation was begun (Harvard Large Animal Ventilator, tidal volume, 10 ml/kg body weight). Sodium heparin (6,000 units, Elkins-Sinn) was administered by intravenous bolus and animals were sacrificed (pentobarbital 100 mg/kg, IV) 20 minutes later. Mechanical ventilation was continued throughout the perfusion procedure. A median sternotomy was performed and the pericardium opened without entering the pleural spaces.

The superior vena cava, inferior vena cava, and ascending thoracic aorta were ligated. The left atrial appendage was opened and a #40 french Sarns catheter (Sarns, Inc.) was positioned at the confluence of the pulmonary veins. The catheter was secured in the left atrial appendage using a purse string suture. A 2 cm transmural incision was made in the right ventricle proximal to the pulmonary outflow tract and a #32 french Sarns catheter was positioned in the main pulmonary artery proximal to its bifurcation. Perfusion catheters were secured by umbilical tape and sterile tubing to capture pulmonary effluent was attached to the left atrial catheter.

All solutions were prepared using aseptic technique. Reagents were obtained from Sigma (St Louis, MO), dissolved and filter sterilized (0.22 μ m) prior to mixing in intravenous infusion bags (Baxter/Travenol) containing sterile NaCl (0.9%). The following solutions were prepared as previously described: (1) 0.01% EDTA, 1 liter; (2) 0.075% sodium nitrate, 500 ml; (3) 0.055% calcium chloride, 1 liter; (4) 0.025% collagenase type IA plus 0.055% calcium chloride, 1 liter.

Perfusion of the pulmonary vasculature was performed by placing fluid bags 30 inches above the pulmonary artery. Infusion fluids were administered in the following order: (1) EDTA, 500 ml; (2) NaNO_3 , 500 ml; (3) EDTA, 500 ml; (4) CaCl_2 , 500 ml; (5) collagenase/ CaCl_2 , 1000 ml; (5) CaCl_2 , 500 ml. Pulmonary effluent obtained prior to infusion of collagenase/ CaCl_2 solution was discarded. The remaining effluent was collected into iced, sterile 250 ml centrifuge tubes (Corning).

Lung perfusion effluent was centrifuged (400 g, 4°C, 15 minutes) and resulting cell pellets washed x 2 in phosphate buffered saline without Ca^{++} or Mg^{++} (PBS, GIBCO,

Grand Island NY), containing 4% Penicillin/Streptomycin (P/S, GIBCO). Cells were resuspended in PBS containing 1% P/S, layered over a 60% Percoll® cushion (Pharmacia), and centrifuged (1000 g, 22°C, 10 min). Following centrifugation, the upper 20% of the gradient was removed and washed in PBS with 1% P/S. Examination of cells in lower regions (i.e., bottom 15%) of the Percoll® gradient revealed mixed cell populations which consisting predominately of neutrophils and lymphocytes with < 5% of cells exhibiting monocyte morphology. The desired cellular fraction was layered over a second gradient Percoll® cushion, centrifuged, and the upper 20% of the gradient removed. Cells were washed twice, resuspended in PBS (1% P/S) and counted manually using a hemacytometer. Viability was determined by Trypan Blue dye exclusion. Differential cell counts were obtained by counting at least 200 cells on modified Wright-Giemsa stained (Dif-Quik®, American Scientific Products, McGaw Park IL) cytocentrifuge preparations (Cytospin 2, Shandon Inc., Pittsburgh, PA).

Cell diameters were measured using a Leitz Orthoplan® light microscope containing an eyepiece reticle. Thirty cells from random fields on Wright-Giemsa stained cytopins were measured at 500X magnification. Results were compared to measurements performed on cytopsin preparations of previously isolated alveolar macrophages and blood monocytes. Results are expressed as mean cellular diameter (μm).

Non-specific esterase staining was performed after the method of Yam et al. Briefly, cytocentrifuge preparations were fixed in a Coplin jar for 15 seconds in Citrate/Acetone/Formaldehyde solution at 22°C with vigorous agitation for the last 5 seconds of incubation. Slides were rinsed in deionized water for 60 seconds and immediately flooded

with a solution containing freshly mixed α -naphthyl acetate, Fast Blue BB Base, Trizmal, and sodium nitrate (Sigma). Slides in this staining solution were incubated in the dark at 37°C for 30 minutes, rinsed with deionized water x 2, counterstained with hematoxylin (2 min), air dried, and preserved under coverslip (Permount). Cells exhibiting blue/black or diffuse tan-brown staining of cytoplasm or cytoplasmic accumulations of brown granules were considered to be non-specific esterase positive. Two hundred cells were graded positive or negative for non-specific esterase staining. Porcine alveolar macrophages and blood monocytes isolated as previously described were used as positive controls.

The ability of harvested cells to re-establish junctional complexes with pulmonary vascular endothelium following removal from the pulmonary microcirculation was determined. Autologous pulmonary artery endothelial explants were prepared by removing a 1 - 2 cm flap of pulmonary artery proximal to the ligation site prior to pulmonary artery cannulation. The pulmonary artery flap was incubated in M199 containing 1% P/S for 2 hours at 37°C. Using sterile technique, the artery was opened, the intimal surface exposed, and small circular endothelial explants created using a sterile 6 mm Baker Biopsy Punch tool (Baker/Cummins). Explants were placed in sterile 6.4 mm tissue culture wells (Costar) containing M199, 5% fetal calf serum, 1% P/S. Cells were adjusted to 10×10^6 cells/ml, 100 μ l aliquots layered over explants and incubated (4 hours, 37°C, 5% CO₂). Following incubation, culture medium was removed and glutaraldehyde (2.5%, pH 7.4) was gently layered over the explants. Following fixation (2 hours at 37°C), explants were placed in PBS and maintained at 4°C until processed for transmission electron microscopy (EM).

Explants were cut into 1 mm x 3 mm pieces. Specimens were post-fixed with 1%

osmium tetroxide for 1 hour at 4°C, followed by dehydration with ethanol and embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, PA). The tissue was embedded in flat embedding molds to facilitate accurate cross sections of the endothelial explant. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were then examined using a Philips EM 400 electron microscope.

Immediately following isolation, cells were adjusted to a concentration of 1×10^6 cells/ml in Dulbecco's modified eagles medium (DMEM), containing 5% calf serum and 1% P/S. Aliquots of cell suspension (300 μ l) were added to separate wells of two sterile tissue culture chamber slides (Lab-Tek). Slides were incubated for two hours at 37°C, 5% CO₂ to permit cell adherence. Following incubation, cell wells were gently washed twice with PBS containing cations. Three hundred μ l of DMEM with or without particulate carbon suspension (200 x dilution of stock india ink suspension) was added to each cell well on both slides. Slides were incubated for 1 hour at either 4°C or 37°C, washed x 4 with PBS, air dried, stained with Dif Quik® and preserved under coverslip. Two hundred cells from random fields were counted on slides incubated under both temperature conditions. Cells demonstrating uptake of 2 or more carbon particles were considered phagocytic. Results are expressed as a phagocytic index established by dividing phagocytic cells by the total cells counted.

RESULTS

DELAYED IBUPROFEN STUDIES.

Study groups included Con (n=5), IbuCon (n=4), Ps (n=10) and IbuPs (n=9) animals respectively. All animals weighed between 15 and 25 kg with a mean weight of 22.1 ± 1.4 kg and no significant differences between the groups. Animals in group Ps exhibited rapid onset of pulmonary arterial hypertension, peaking at 49 ± 2 mmHg within 30 minutes of commencing infusion of live *Pseudomonas*. Pulmonary pressures moderated following the peak at 30 min but remained significantly elevated above both group Con and IbuCon for the duration of the study ($p < 0.05$). Control animals receiving an infusion of ibuprofen (IbuCon) demonstrated no significant increase in PAP, over the 5 hr study period, when compared to baseline measurements. Infusion of ibuprofen into septic animals (IbuPs) resulted in a significant attenuation of early onset pulmonary artery hypertension. Subsequently, a gradual rise in PAP occurred, resulting in levels equivalent to those in Ps animals at 90 and 120 min. Infusion of ibuprofen at 120 min again attenuated PAP. However, from 210 min to 300 min PAP levels reached those observed in unprotected septic animals (Ps). Systemic arterial pressure in group Ps dropped significantly by 60 minutes below controls (Con, IbuCon) with significant hypotension persisting to 300 minutes. Ibuprofen administered to septic animals maintained SAP at control levels throughout the study. In Ps animals, PaO_2 fell below control levels shortly after the outset of the study, becoming significantly depressed at 60 min. No changes in PaO_2 from baseline was observed in either group Con or group IbuCon. Infusion of ibuprofen into septic animals (IbuPs) maintained PaO_2 at control levels until 300 min. Cardiac index fell progressively in group

Ps with significant depression compared to groups Con and IbuCon evident from 120 min onwards. Cardiac index in group IbuPs was indistinguishable from group Ps for the duration of the study. As previously documented in this septic porcine model, PAOP remained at baseline levels in all groups until 300 min.

ALVEOLAR CAPILLARY MEMBRANE PERMEABILITY.

Thermal-cardiogreen extravascular lung water (EVLW). Extravascular lung water measurements in the control animals and control animals receiving ibuprofen remained at baseline levels throughout the study (Fig 1). A sustained rise in EVLW in group Ps animals was observed. Statistical significance became apparent at 120 minutes, reaching a maximum of 16.2 ± 1.7 ml by 300 min ($P < 0.05$ vs Con, 120-300 min). Group IbuPs exhibited a gradual rise in EVLW, reaching a peak of 10.1 ± 1.2 ml at 180 min with a subsequent fall to 8.9 ± 1.0 at 300 min. Extravascular lung water in IbuPs animals was significantly less than that observed in Ps animals at 240 and 300 min. At no time were EVLW levels significantly elevated above control values in group IbuPs ($p > 0.05$ 0-300 min).

BRONCHOALVEOLAR LAVAGE PROTEIN CONTENT (BAL-P). Returns of bronchoalveolar lavage fluid were consistently high, between 75 and 79% in all animals in this study. A significant increase in BAL protein content was observed in group Ps animals at 300 min (Fig 2), BAL protein content in group Ps was 5.2 times that observed at baseline in the same group (0min, 130 ± 22 μ g/ml vs 300 min, 683 ± 133 μ g/ml, $p < 0.01$). In contrast, there was no protein accumulation in the airspace over 5 hrs in group Con animals. Delayed ibuprofen (group D-Ibu) treatment in septic animals prevented accumulation of protein at 300 min (Fig 2).

POLYMORPHONUCLEAR LEUKOCYTES.

Circulating and BAL PMN counts: Baseline peripheral PMN counts for all animals in this study was $23.4 \pm 1.6 \times 10^3$ cells/ μ l (range 17.0 - 31.5×10^3 cells/ μ l). Total PMN count remained at baseline levels throughout the 300 min of study in the control (Con) animals as well as in the group IbuCon animals. In septic animals (Ps), total circulating PMN counts dropped soon after the *Pseudomonas* infusion. Significant neutropenia was well established within 30 minutes and persisted to the end of the study. Similarly, septic animals treated with ibuprofen (IbuPs), exhibited rapid onset neutropenia paralleling that in septic unprotected animals (Fig 3)

All groups had minimal PMNs in the airspace under normal conditions. A neutrophilic alveolitis was evident in group Ps at 300 min (24%) and was abolished in group D-Ibu.

SUPEROXIDE ANION GENERATION (IN-VIVO).

There was no spontaneous generation of O_2^- from PMNs at any of the experimental timepoints in groups Con or IbuCon. The maximal rate of O_2^- production (nmol O_2^- /10⁶ PMN/min) occurred approximately 4 minutes following PMA stimulation at each time point: 3.7 ± 0.4 (0 min), 3.2 ± 0.3 (60 min) and 3.5 ± 0.6 (300 min) $p = 0.19$ ANOVA. Thus, in controls, no differences were observed between PMN O_2^- generation pre or post saline infusion or at 300 min. In control animals receiving infusions of ibuprofen there was a trend towards lower maximum levels of O_2^- generation when compared to similar timepoints in group Con however, these values were not significantly different. In contrast, PMA-stimulated PMNs from group Ps demonstrated significantly increased O_2^- generation at 60 min with a further dramatic increase by 300 min ($p < 0.05$ ANOVA) when compared to O_2^-

generation observed in PMNs pre-*Pseudomonas* infusion. The maximal rate of O_2^- production from "septic" PMNs at 60 and 300 min occurred at 2-3 minutes following PMA stimulation. Quantitatively, "septic" PMN obtained from group Ps animals exhibited a maximal O_2^- production rate of 4.1 ± 0.2 nmol/min at 60 min and 6.0 ± 0.5 nmol/min at 300 min (Fig 4). These values occurred at the two minute timepoint following PMA stimulation and were significantly higher, $p < 0.01$ and $p < 0.001$, respectively, than that observed in 60 min and 300 min control PMNs (Con,IbuCon). Thus, cells remaining in circulation following the onset of sepsis exhibited dramatic "priming " for short-lived oxidant production.

EFFECT OF CYCLO-OXYGENASE INHIBITION ON SUPEROXIDE ANION PRODUCTION.

When the continuous production of O_2^- from PMNs in group IbuPs at the 0, 60 and 300 min timepoints was compared, analysis of variance demonstrated no differences between the O_2^- generation of these PMN samples. The kinetic curves described by each of the three PMN samples were quite similar to those produced by PMNs from control animals. Unlike PMNs from septic unprotected animals (group Ps), maximal rates of O_2^- generation occurred 4 min following PMA stimulation. Although there was a trend for increased O_2^- generation in the PMNs harvested at 60 and 300 min compared to baseline in group IbuPs, this was not statistically significant. Therefore, the priming response noted in the septic unprotected animals was abolished.

ENDOTHELIAL CELL LINE AND FLOW CYTOMETRY STUDIES.

Neutrophil CD18 expression was not altered in control animals. In septic animals, a significant increase in CD18 expression occurred. This increase was maximal at 120 min after the onset of sepsis, a time which coincided with the lowest recorded peripheral neutrophil counts (Fig 5). The degree of expression was reduced after 180 min but remained significantly greater than controls throughout the study period.

Measurement of peripheral neutrophil CD18 expression at 300 min and comparison with neutrophils that had migrated into the alveolus revealed a significant increase in CD18 expression after passage across the alveolar capillary membrane (Fig 6). These data implicate the CD18 adhesion receptor as an important mechanism in the migration of neutrophils from the vascular to alveolar spaces during acute septic lung injury. Preliminary data using the monoclonal antibody 60.3 as a therapeutic agent support this hypothesis (Fig 7). The dramatic decline in peripheral neutrophil count was reduced as was the migration of neutrophils across the alveolar capillary membrane (Fig 8).

In vitro studies incubating neutrophils with TNF show that TNF is responsible for significant increases in CD18 expression which occurred in a time and dose dependent fashion (Figs 9,10). Correlative in vivo studies measuring serum TNF levels and neutrophil CD18 levels suggest that TNF might be the factor responsible for increased CD18 expression in sepsis and may be an important mediator of the profound neutropenia seen in this model.

TUMOR NECROSIS FACTOR (TNF) STUDY.

No significant TNF activity was detected in control pig plasma (Fig 11). In septic pigs, TNF activity appeared in plasma 15 min after the onset of sepsis and remained elevated throughout the study period (Fig 11). The appearance of pulmonary arterial hypertension, increased lung water and deteriorating gas exchange correlated with the rise in plasma TNF activity which peaked at 90-120 mins in septic pigs. Our results provide evidence that both $\text{TNF}\alpha$ and $\text{TNF}\beta$ are present in plasma in sepsis (Figs 12,13,14). Anti $\text{TNF}\alpha$ and $\text{TNF}\beta$ neutralized TNF activity in whole septic plasma at 15, 30 and 45 min following onset of septicemia and the antibodies blocked TNF activity in serially diluted septic plasma at all time points up to 210 min of sepsis. TNF activity was not neutralized entirely by TNF antisera from 210-300 min in septic plasma. These results suggest that $\text{TNF}\alpha$, $\text{TNF}\beta$ and other "TNF like" activity increases in plasma during septicemia and that this elevated TNF activity correlates with the development of acute lung injury in the pig.

No TNF activity was detected in non-septic pigs and only slight TNF activity was detected in non-septic animals given ibuprofen. In pseudomonas infused animals, plasma TNF activity rose by 30 min and remained elevated throughout the study, while septic pigs given ibuprofen exhibited decreased TNF activity. In other experiments to determine whether delaying the ibuprofen treatment would have the same effect, ibuprofen was administered at 30 and 120min after the onset of sepsis. Delaying the treatment did not affect the marked decrease in TNF levels seen with ibuprofen pretreatment (Fig 15).

PULMONARY INTRAVASCULAR MACROPHAGE STUDIES

Twenty-six pigs were studied by the technique described. Of the collectable 1.5 L volume, 924 ± 84 ml were recovered following in-situ lung perfusion. Preliminary studies to determine the optimum collagenase concentration for cell recovery were performed by varying the collagenase between 0.01 and 0.1%. Infusion of high concentrations of collagenase (0.1%), as previously described, rapidly produced visible disruption of intact lung with accompanying edema and resulted in diminished flow of left atrial effluent (556 ± 40 ml, $n=4$). Infusion of reduced collagenase concentrations (i.e., 0.01% - 0.035%, $n=20$) preserved effluent flow (1122 ± 124 ml) with little visible lung disruption. No correlation between perfusate collagenase concentration and cell yield was observed (Figure 2), ($r = 0.3$, NS). Mean total cell numbers obtained in this study was $3.8 \pm 0.5 \times 10^8$ cells. Cell viability was consistently high ($> 98\%$) and was not altered by changes in collagenase concentration (data not shown). After preliminary experiments all further animals were perfused at 0.025% collagenase concentration.

MORPHOLOGICAL AND HISTOCHEMICAL CHARACTERISTICS OF RETRIEVED CELLS.

Heterogenous mononuclear cells populations were obtained from the microcirculation of porcine lung. (Figure 3). Most of the cells ($71 \pm 1\%$) were small mononuclear cells with indented nuclei and reduced cytoplasmic volume (diameter $10.4 \pm 0.27 \mu\text{m}$). Larger mononuclear cells with irregular, asymmetric nuclei comprised $25 \pm 1\%$ (diameter $16.5 \pm 0.31 \mu\text{m}$) ($p < 0.05$, large vs small mononuclear cells) of observed cells while lymphocytes and neutrophils were 1% and 2% respectively. Porcine alveolar macrophages and blood monocytes exhibited mean cellular diameters of $21.4 \pm 0.5 \mu\text{m}$ and $10.5 \pm 0.22 \mu\text{m}$

respectively. Cellular morphology of cell populations obtained were unaffected by the concentration of collagenase employed in the isolation procedure. Greater than 87% of isolated cells adhered to tissue culture plastic following a 1 hr incubation at 37°C. Morphology and relative composition of 1 hr adherent cells remained constant compared to initial cell isolates. When allowed to remain in culture for 7 days (DMEM, 5% calf serum, 37°C, 5% CO₂) cells exhibited increased cell size and cytoplasmic volume (Figure 4). Cells isolated by the technique re-established intercellular adhesion plaques on explanted endothelial surfaces (Figure 5).

Esterase positive staining was observed in $73\% \pm 5\%$ of cells ($n=4$). However, staining characteristics differed somewhat from that observed in porcine alveolar macrophages. Both large and small cell populations exhibited diffuse uptake of α -naphthyl acetate resulting in homogenous tan/brown staining of cells. Many cells exhibited stippling throughout with fine esterase positive granules. Plasma membrane esterase activity detectable as black "rim like" staining typical of the monocyte/macrophage lineage was identified in both cell populations (Figure 6) (21). In contrast, alveolar macrophages exhibited heavier staining concentrated in coalesced granular areas. Porcine blood monocytes exhibited similar, though less intense, staining to that observed in mononuclear phagocytes obtained by lung perfusion. Lymphocytes and neutrophils identified on cytospin preparations failed to take up the non-specific esterase stain.

Mononuclear cells obtained by this technique phagocytosed particulate carbon in-vitro and exhibited a phagocytic index of $80 \pm 6\%$ at 37°C. Cells exposed to identical particulate loads at 4°C exhibited a phagocytic index of $9.5 \pm 4\%$ ($P < 0.01$). No evidence of particle

uptake was observed in cells incubated without particulate carbon. Both large and small constituent members of harvested cell populations phagocytosed particulate carbon.

CONCLUSIONS.

The lung injury in ARDS is mediated by a number of different cell types as well as a multitude of biological mediators, some of which may yet be unknown. Histamine, prostaglandins, oxygen free radicals, small molecular weight biologically active lipids, interleukins, tumor necrosis factor and proteolytic enzymes have been implicated in the injury. We have already established in the porcine pseudomonas model that treatment with a combination of H_1 and H_2 blockers and a cyclooxygenase inhibitor can attenuate all phases of the injury, particularly the early phase of pulmonary hypertension, as well as the late permeability phase.

We have established that cyclooxygenase inhibitors prevented the rise in TxB_2 and PAP. It appears that thromboxane plays an important role in PAP mediated endotoxin and pseudomonas lung injury. Importantly, we have shown that delayed treatment with cyclooxygenase inhibitors is also effective.

It is becoming clear from in vitro work that oxygen radicals do have a role in the injury, but how this can be prevented is not apparent. The administration of exogenous radical scavengers in this model system has not altered the injury in any significant way. It may be that the best way to prevent oxygen radical induced injury is to prevent neutrophil adhesion to pulmonary endothelium, thus preventing the formation of an impenetrable microenvironment where endogenous antioxidants cannot neutralize the damaging effects of oxygen radicals. The indirect methods used in this laboratory and outlined in this report show that the potential of circulating phagocytes to cause cellular damage can be adequately assessed.

We have established in this model that pseudomonas "primed" neutrophils produce superoxide radicals at a much greater rate than in control animals, thus implicating neutrophils as a major injury cell in the early phase of the process. However, it appears from preliminary results that alveolar macrophages may also be implicated in the injury but, because of their position, priming of these cells takes longer than neutrophils. It may be that macrophages, in addition to producing interleukins and tumor necrosis factor, also produce superoxide and its metabolites and may be involved in the later stages of alveolar-capillary injury. Production of both short and long lived oxidants are elevated in septic animals and this elevation is attenuated to a large extent by delayed treatment with ibuprofen.

Tumor necrosis factor increases significantly shortly after the onset of pseudomonas infusion in the model and probably plays an important role in the early phase of the injury. This increase is also attenuated by delayed treatment with ibuprofen. No therapy to date has been able to prevent the dramatic neutropenia seen in this model. Our results suggest that this may be a CD18 dependent phenomenon and as such may respond to monoclonal antibody treatment which prevents neutrophil adhesion. Preliminary data suggest that this is indeed the case. The in vitro studies looking at TNF and neutrophil CD18 expression suggest that TNF may be in part responsible for this effect in vivo. This hypothesis will be tested when we look at the effect of an anti TNF monoclonal antibody as a form of therapy in the model.

The technique we have developed for harvesting PIMs should enable further elucidation of the role of this cell line in ARDS as this harvest technique allows large

numbers of cells to be studied ex vivo. Only after these ex vivo studies have been performed will we have a better idea of the true importance of this cell line in the pathogenesis of ARDS.

RECOMMENDATIONS

ARDS still carries a considerable mortality in both civilian and military patients, especially if associated with the sepsis syndrome. It is particularly devastating because it often occurs in previously healthy individuals who sustain either pulmonary or non-pulmonary insults. It has been established that the acute lung injury seen in ARDS is due to a complex interaction of host immune cells and a multitude of inflammatory mediators. Elucidation of cell interactions and mediator pathways will be necessary for appropriate immunological and pharmacological intervention and treatment of the syndrome. We have established, in an animal model, that delayed treatment with a cyclooxygenase inhibitor (ibuprofen) significantly improve many parameters of the syndrome. The presence of significantly elevated TNF levels in this model suggest that the use of immunomodulation (monoclonal antibodies to TNF) may provide important further advances. The correlation of significantly increased neutrophil CD18 expression with the dramatic neutropenia seen in this model, as well as the increased CD18 expression seen on neutrophils that migrate to the alveous, suggests that monoclonal antibody therapy which blocks neutrophil adhesion via a CD18 dependent mechanism may be of value in preventing neutrophil induced alveolar capillary membrane damage.

Our preliminary work with the pulmonary intravascular macrophage (PIM) has convinced us to evaluate this cell in both clinical ARDS and experimental models of acute lung injury.

Publications

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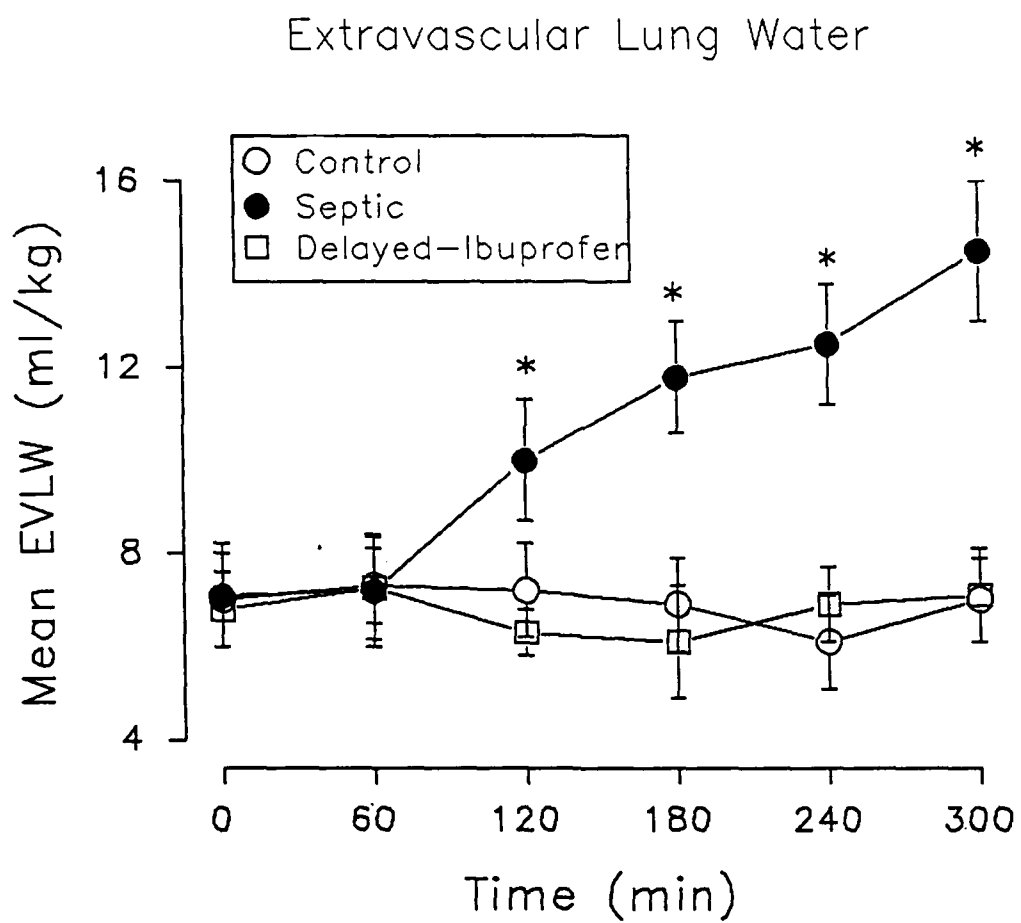
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Figure 1



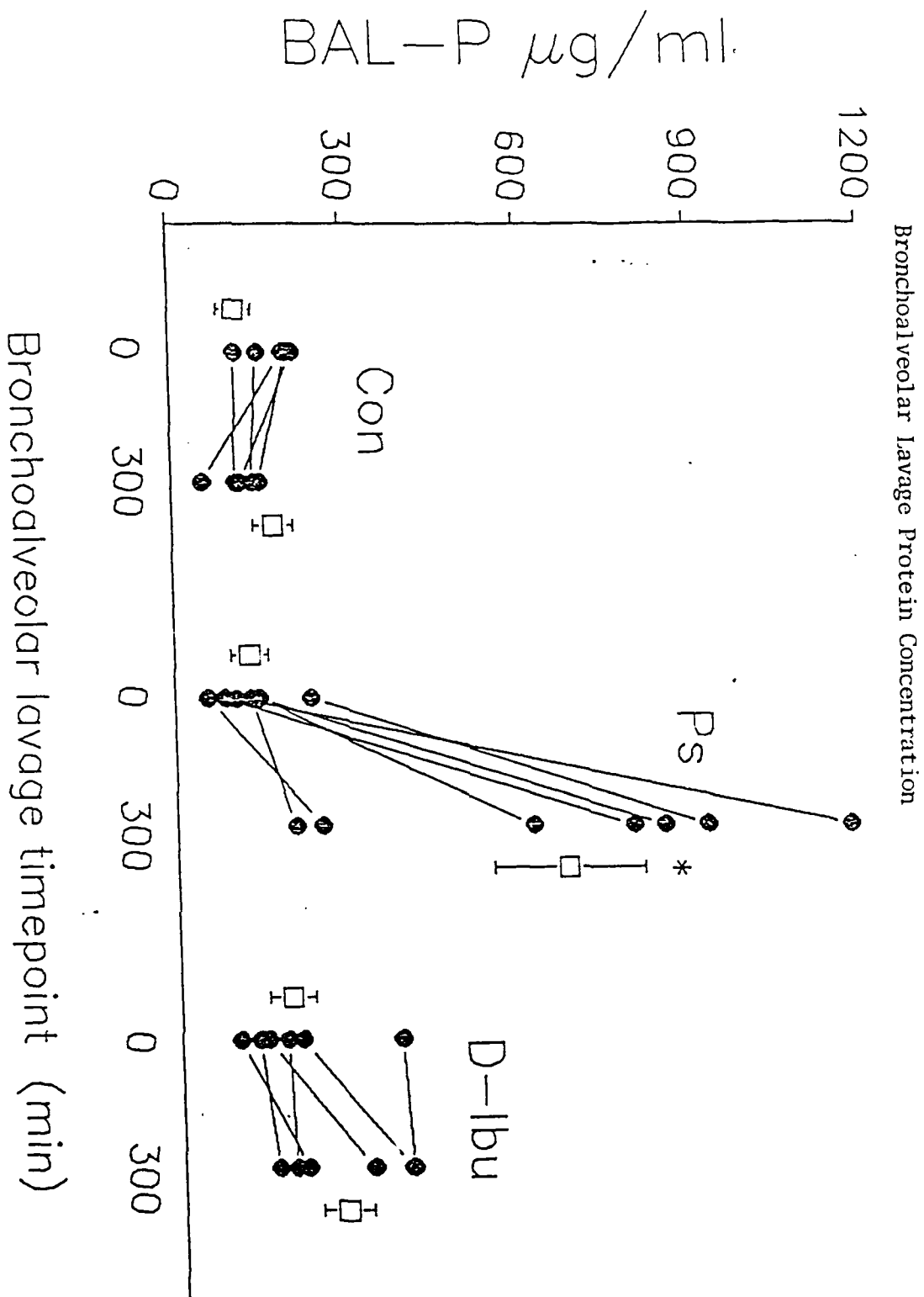


Fig 2

Figure 3

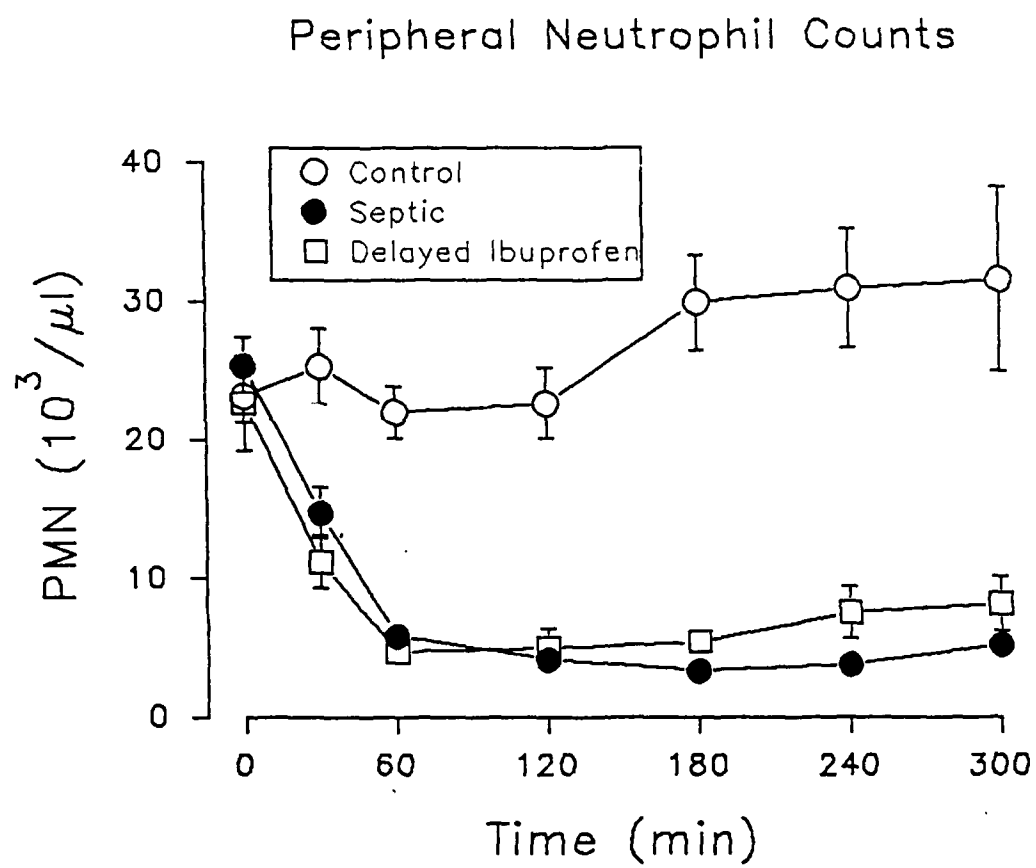


Fig 4 Superoxide Anion Kinetics

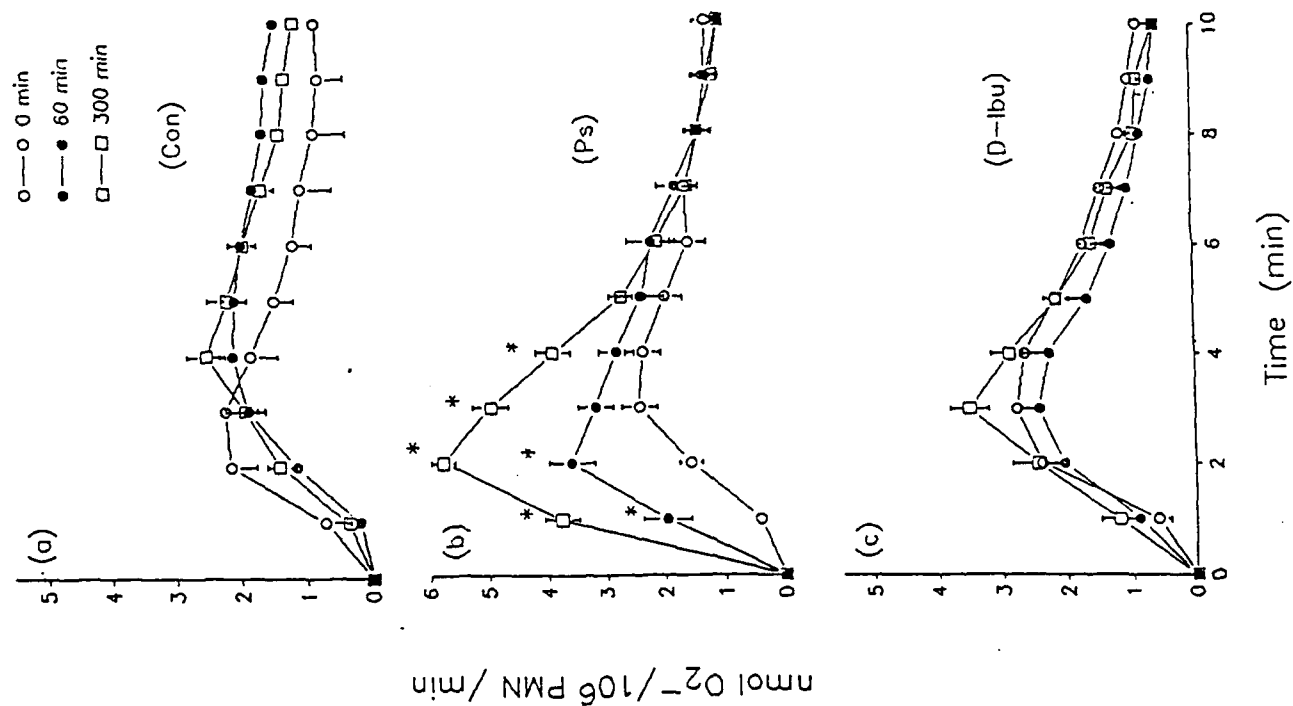


Figure 5

PMN CD18 Expression vs PMN in Sepsis

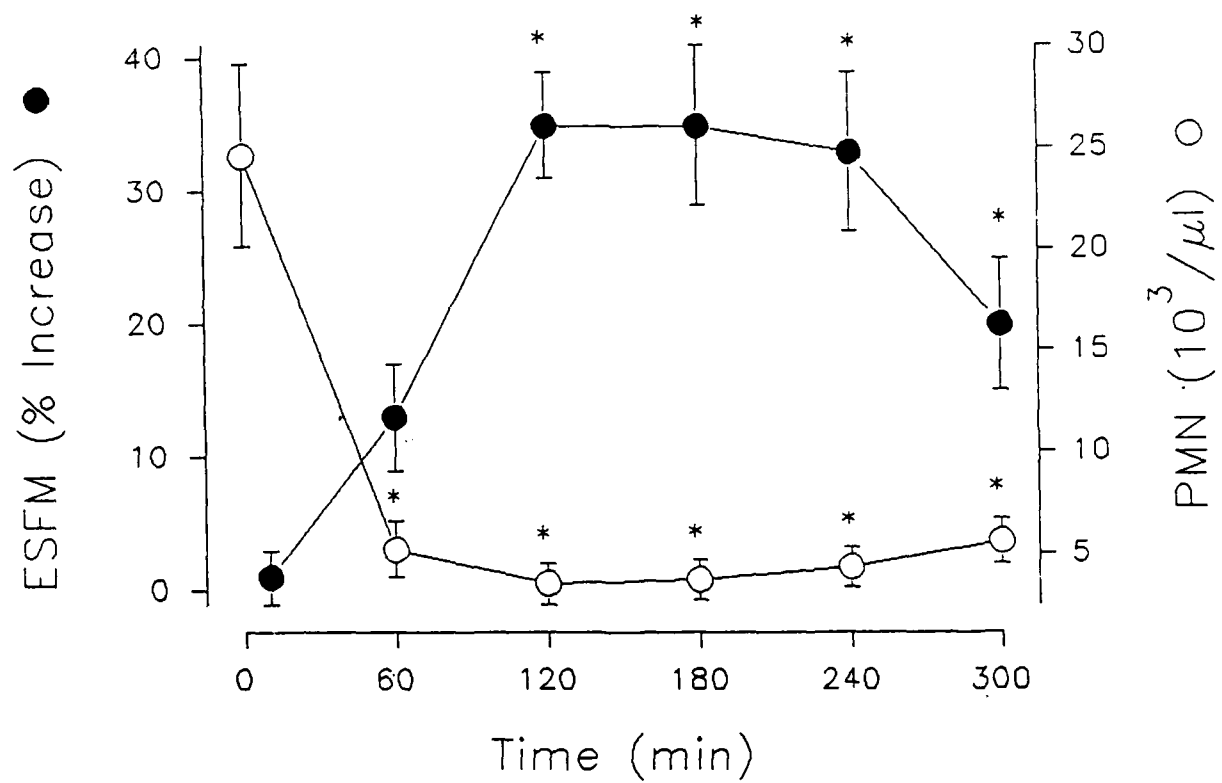


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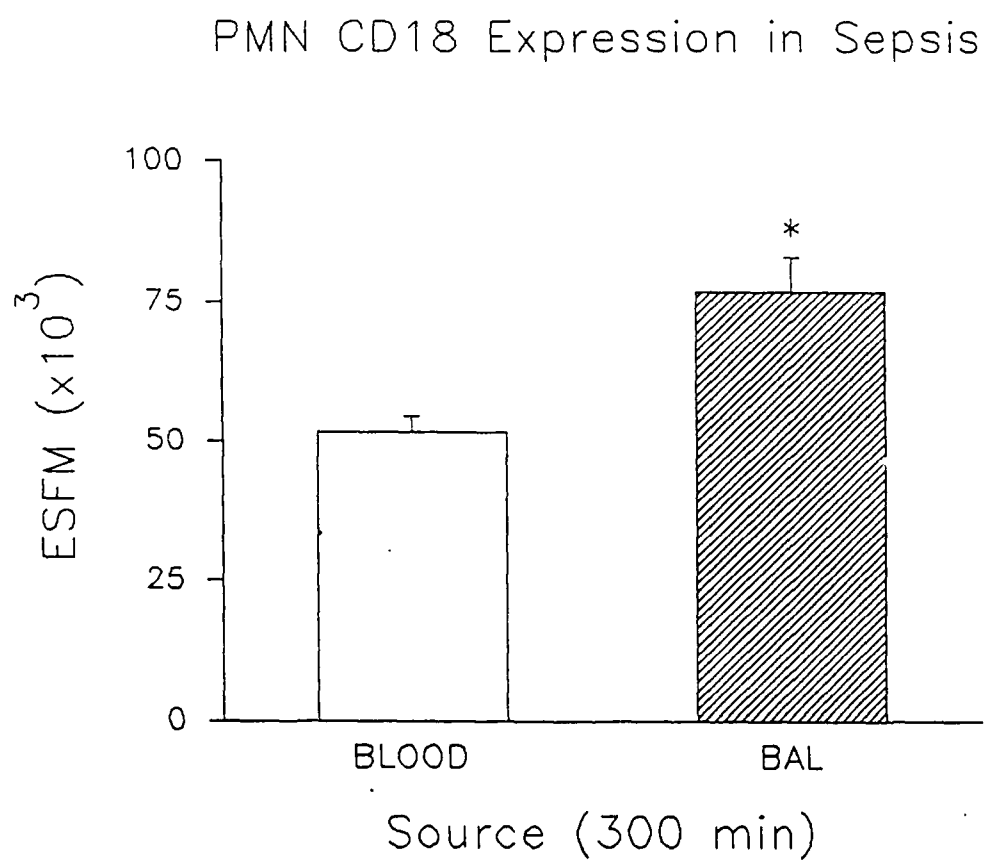


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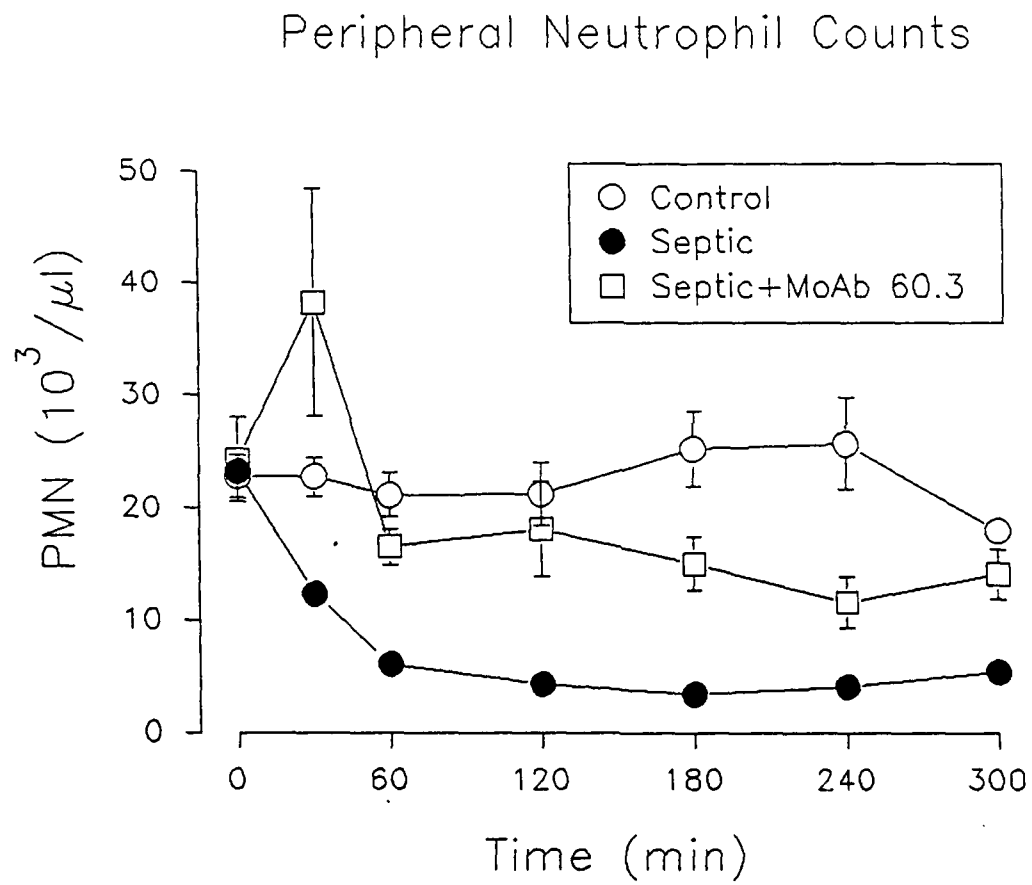


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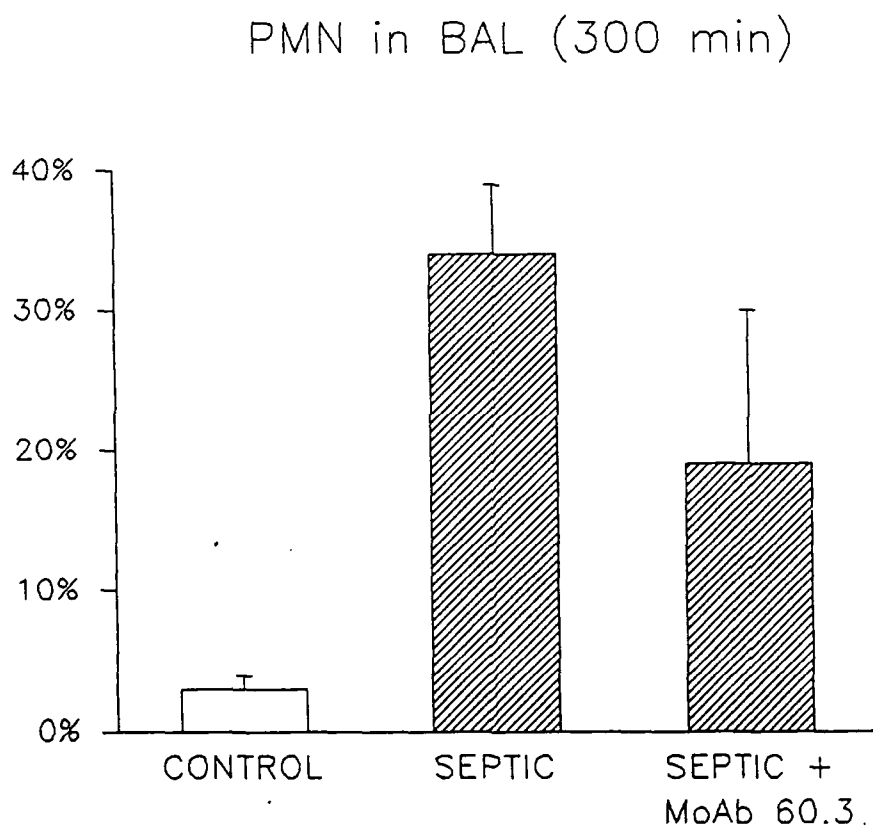


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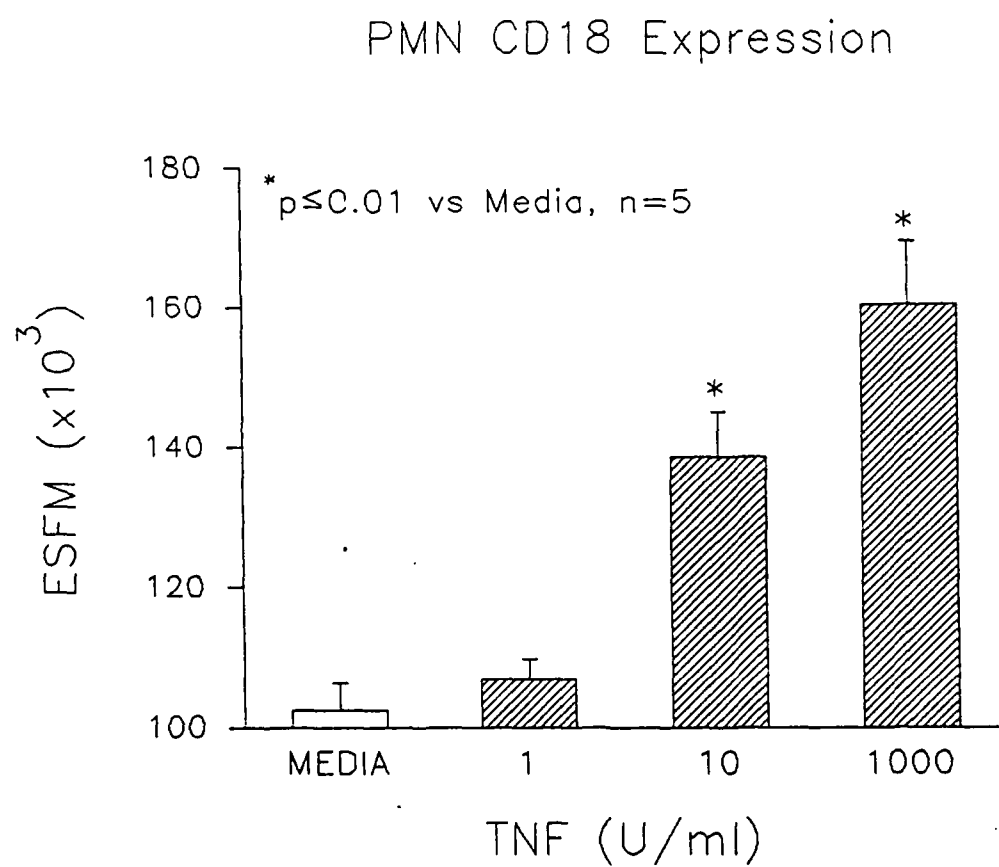


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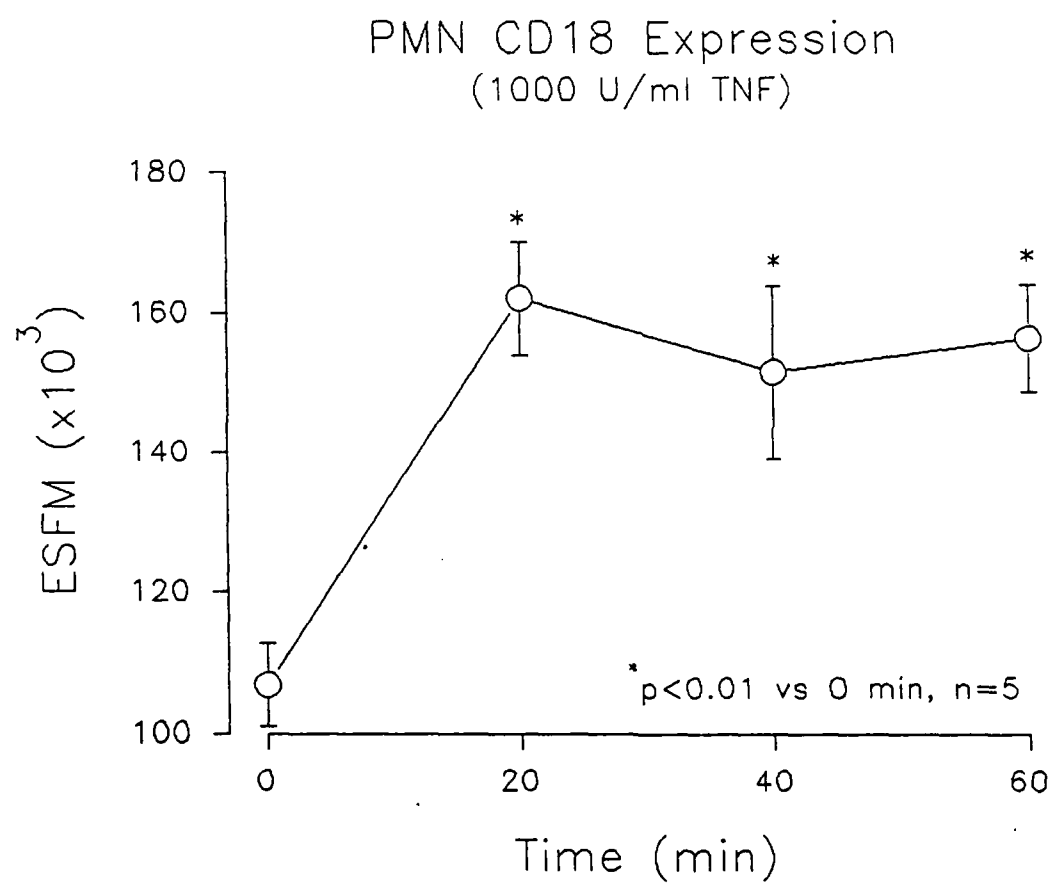


Figure 11

Plasma TNF Levels in Porcine Sepsis

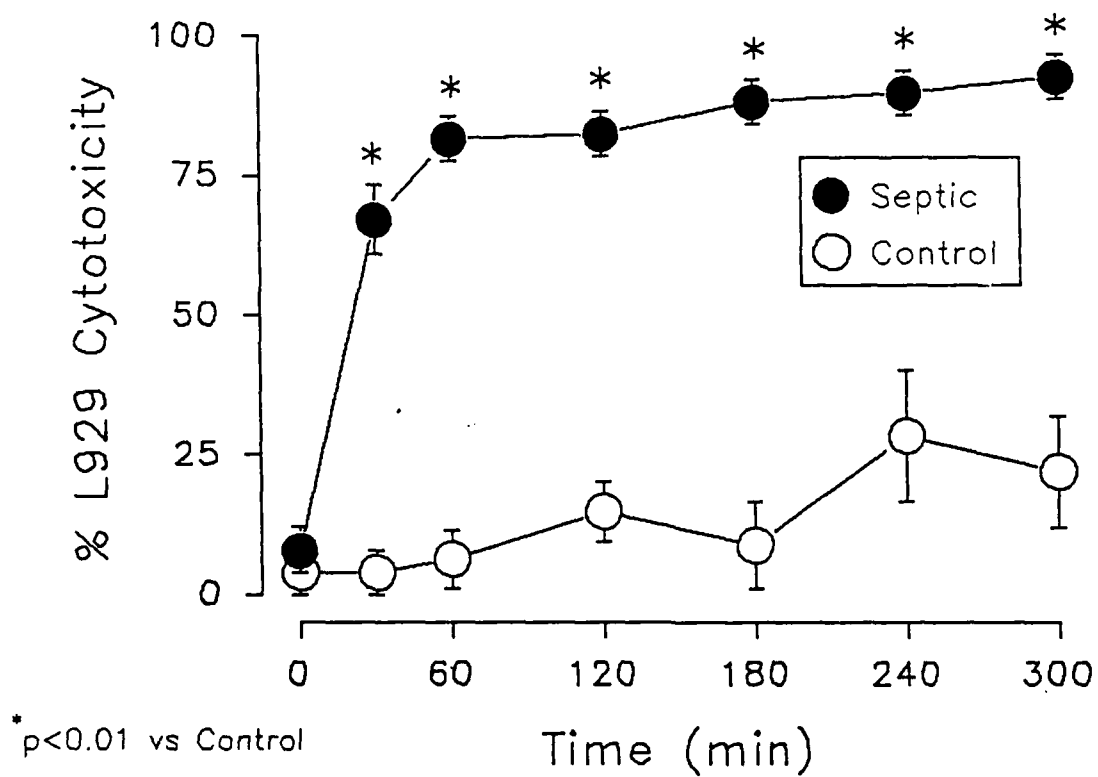


Figure 12

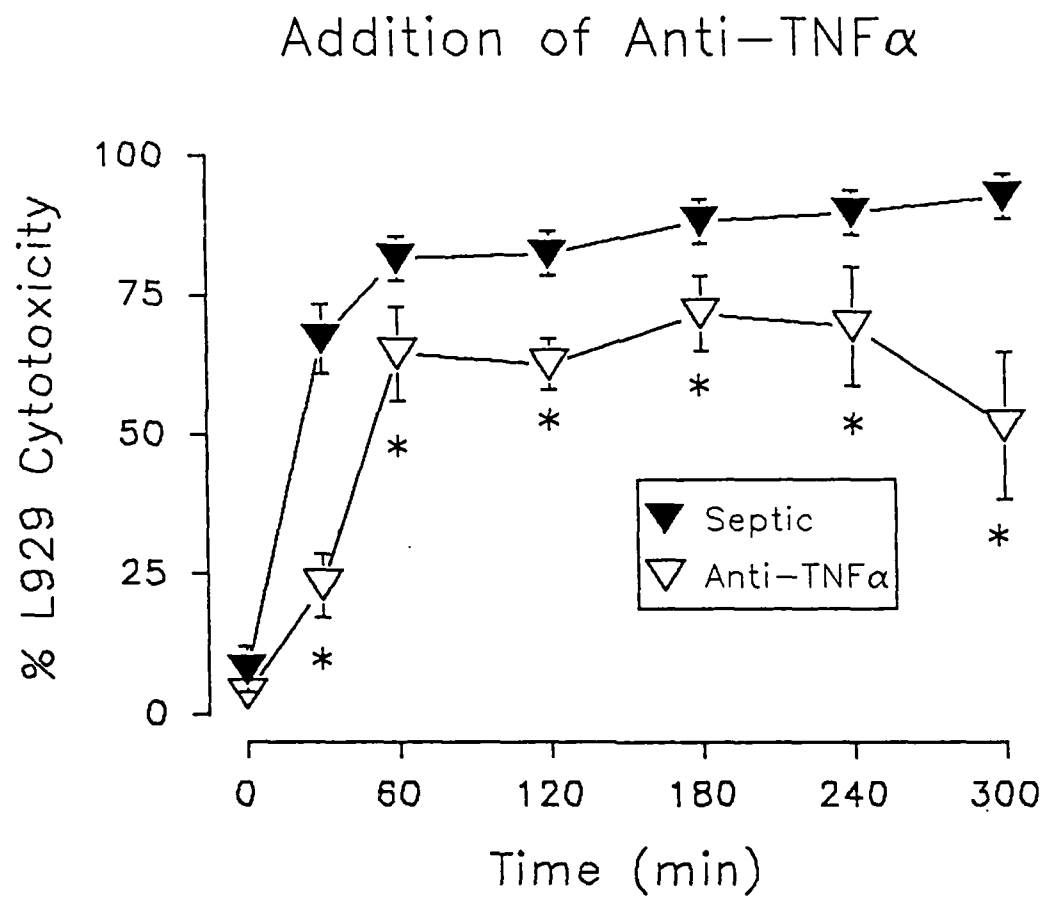


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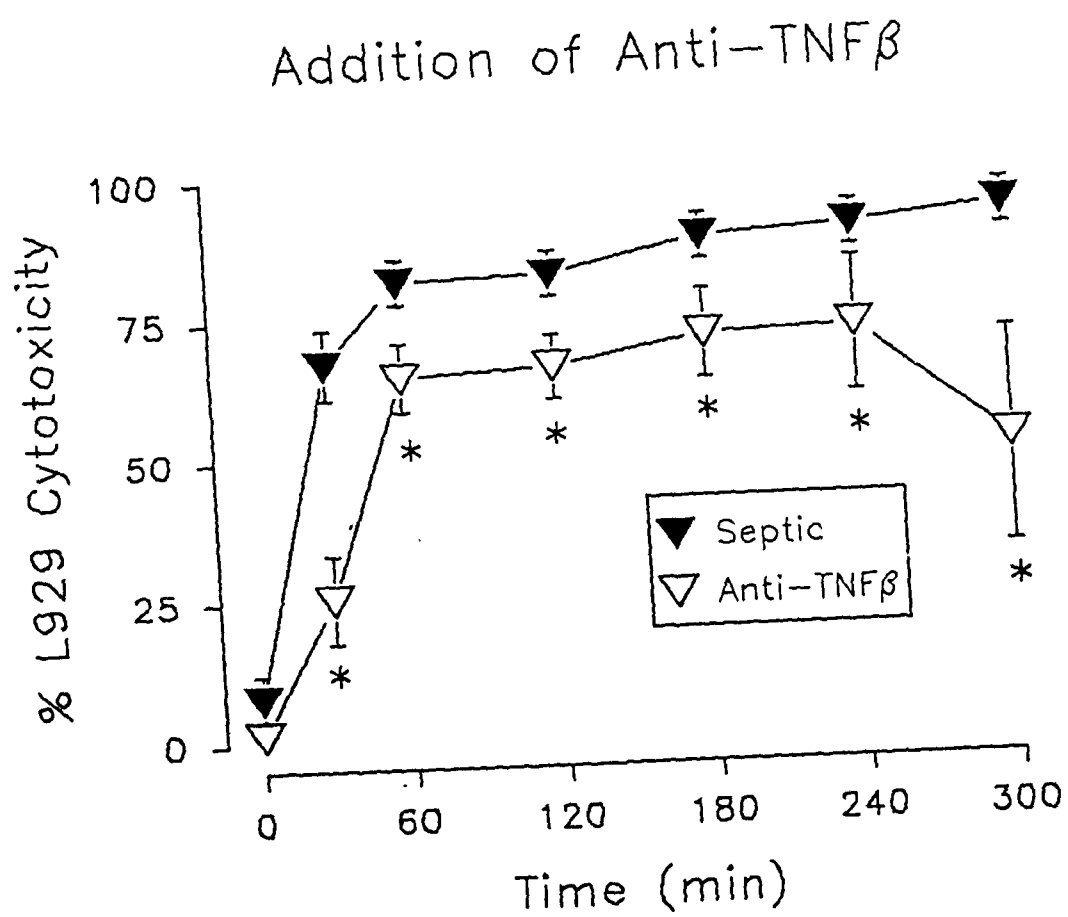


Figure 14

Addition of Anti-TNF α and Anti-TNF β

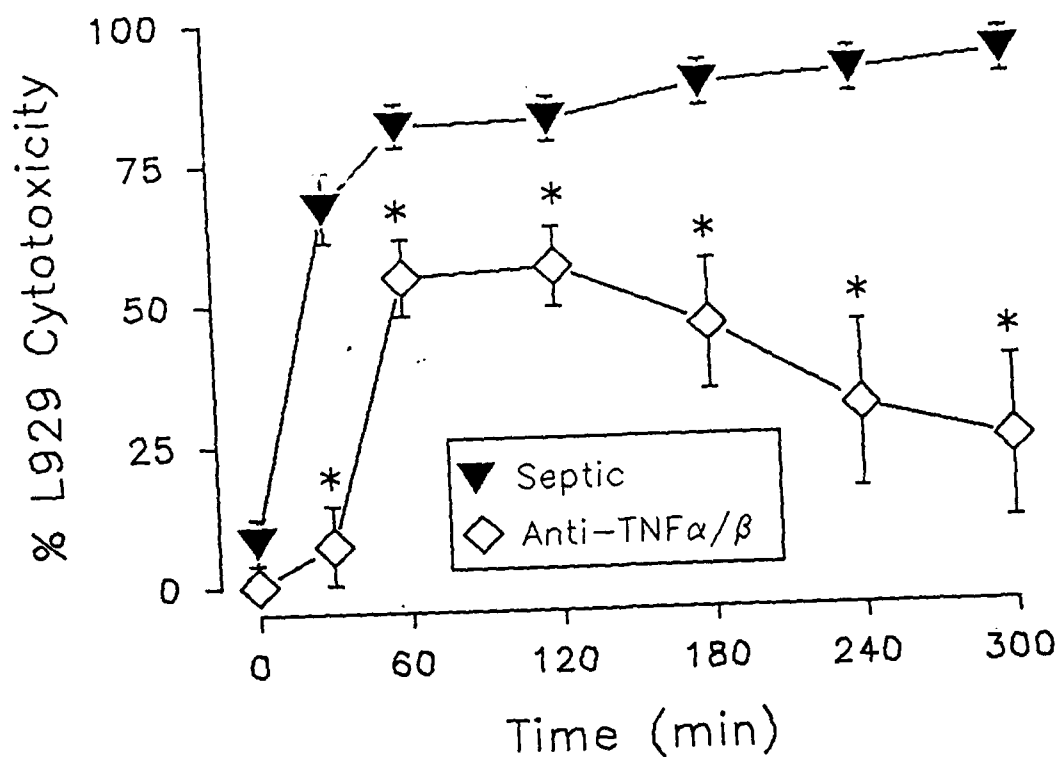


Figure 15

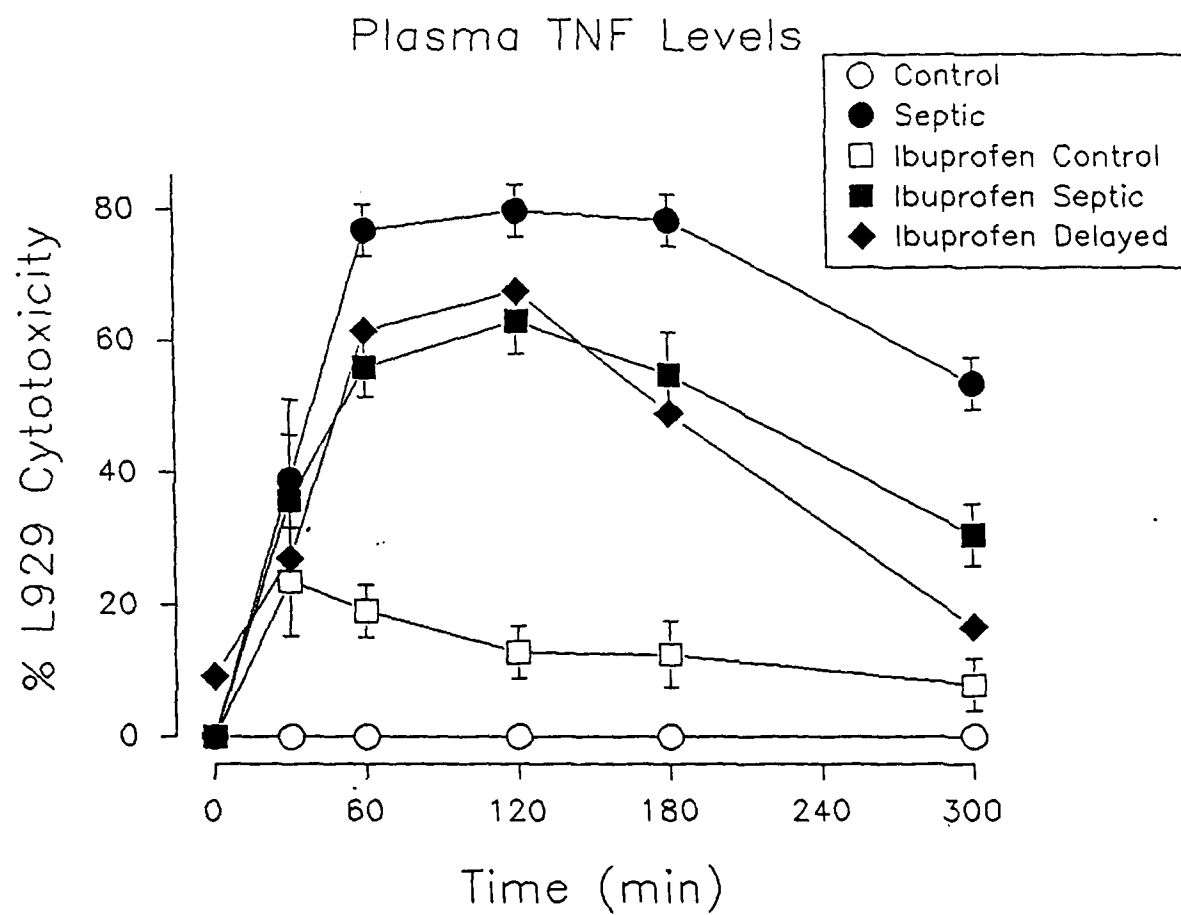


Figure 16. (See reverse)

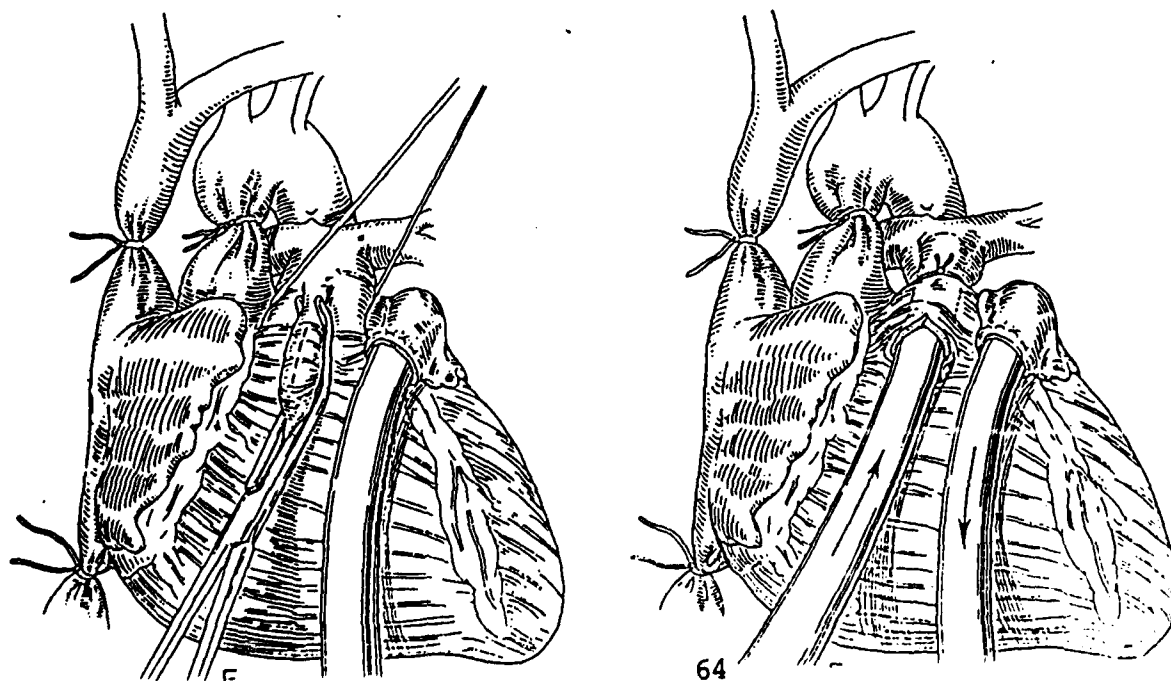
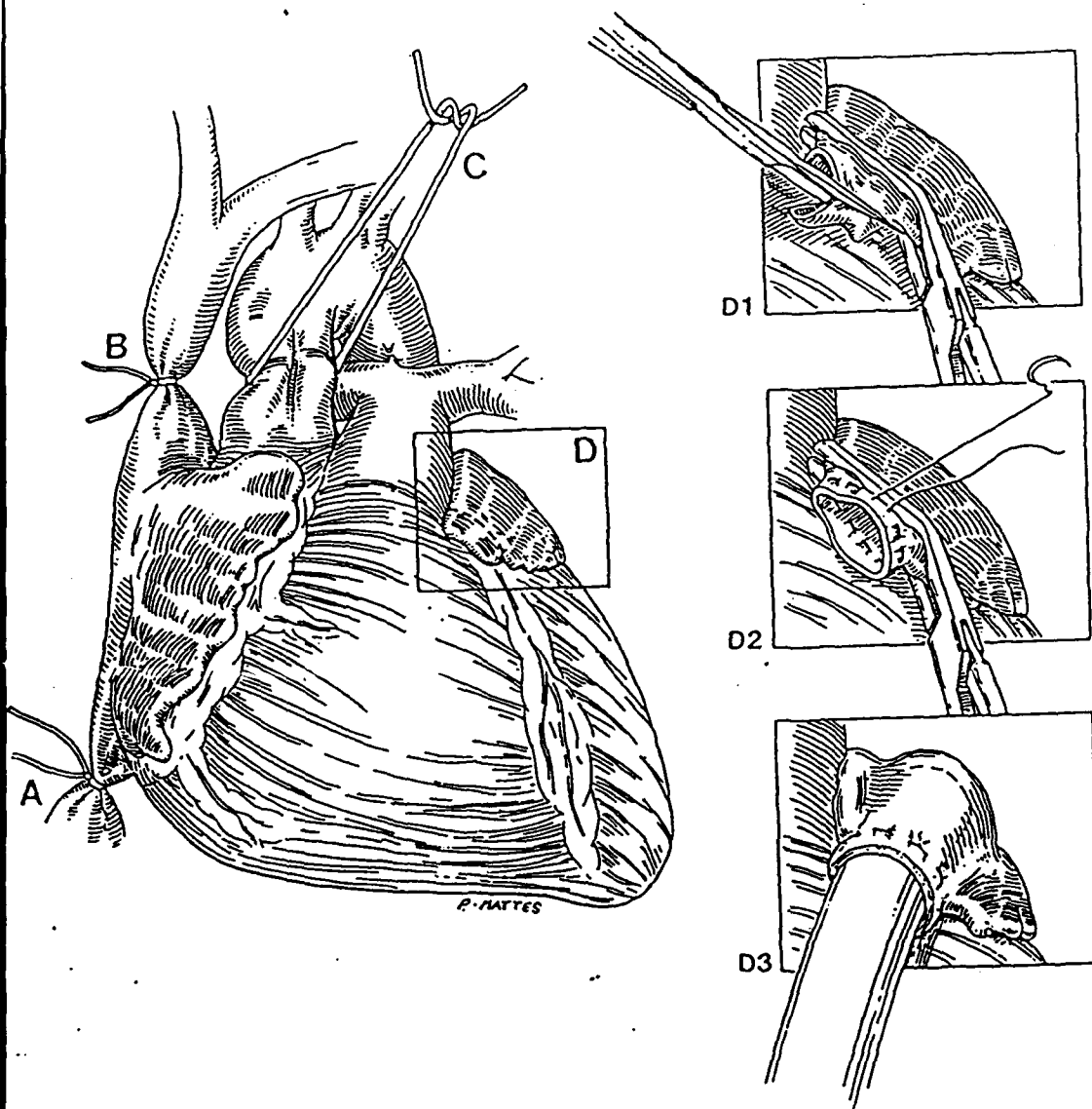


Figure 16. This line drawing outlines the sequence used for the placement of ligatures (A-C) on the great vessels followed by illustrations of the technique for placement of the Sarn's venous return catheters. Following placement of the catheters (D-E), the lung is perfused blood free with EDTA, sodium nitrate, and calcium chloride containing solutions. Once blood free the lung is perfused with a collagenase/calcium chloride solution to remove anchored mononuclear phagocytes from lung microcirculation (see text of material and methods).

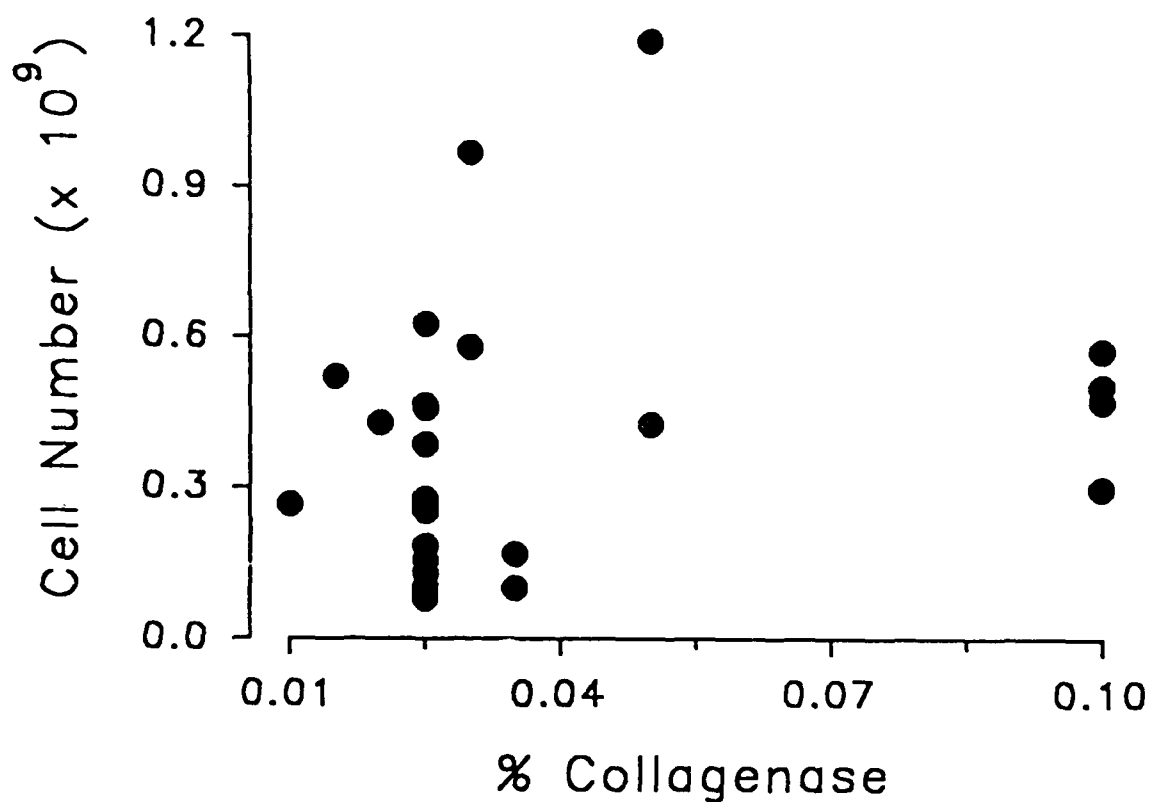


Figure 17. The concentration of enzyme utilized for lung perfusion was plotted against the total numbers of cells retrieved. No correlation could be found between the collagenase concentration and cell number retrieved. The mean total cell yield for all 26 experiments was $3.8 \pm 0.5 \times 10^8$ (\pm SEM) mononuclear cells.

A

B

- Figure 18. A. Freshly isolated mononuclear cells obtained from the porcine pulmonary microcirculation were heterogenous populations consisting of $71 \pm 1\%$ small mononuclear cells (diameter $10.4 \pm 0.27 \mu\text{m}$) with indented nuclei and reduced cytoplasmic volume. Twenty-five $\pm 1\%$ of cells were larger (diameter $16.5 \pm 0.31 \mu\text{m}$) possessing irregular, asymmetric nuclei. Lymphocytes and neutrophils were 1% and 2% of retrieved cells respectively (600X magnification, Wright-Giemsa).
- B. Retrieved mononuclear cells cultured for seven days developed macrophage morphology with nucleus becoming eccentrically located and increases in cytoplasmic volume. Small cell populations increased to $30 \pm 1 \mu\text{m}$ diameter while larger mononuclear cells increased to $46.6 \pm 1.6 \mu\text{m}$ (600X magnification, Wright-Giemsa).

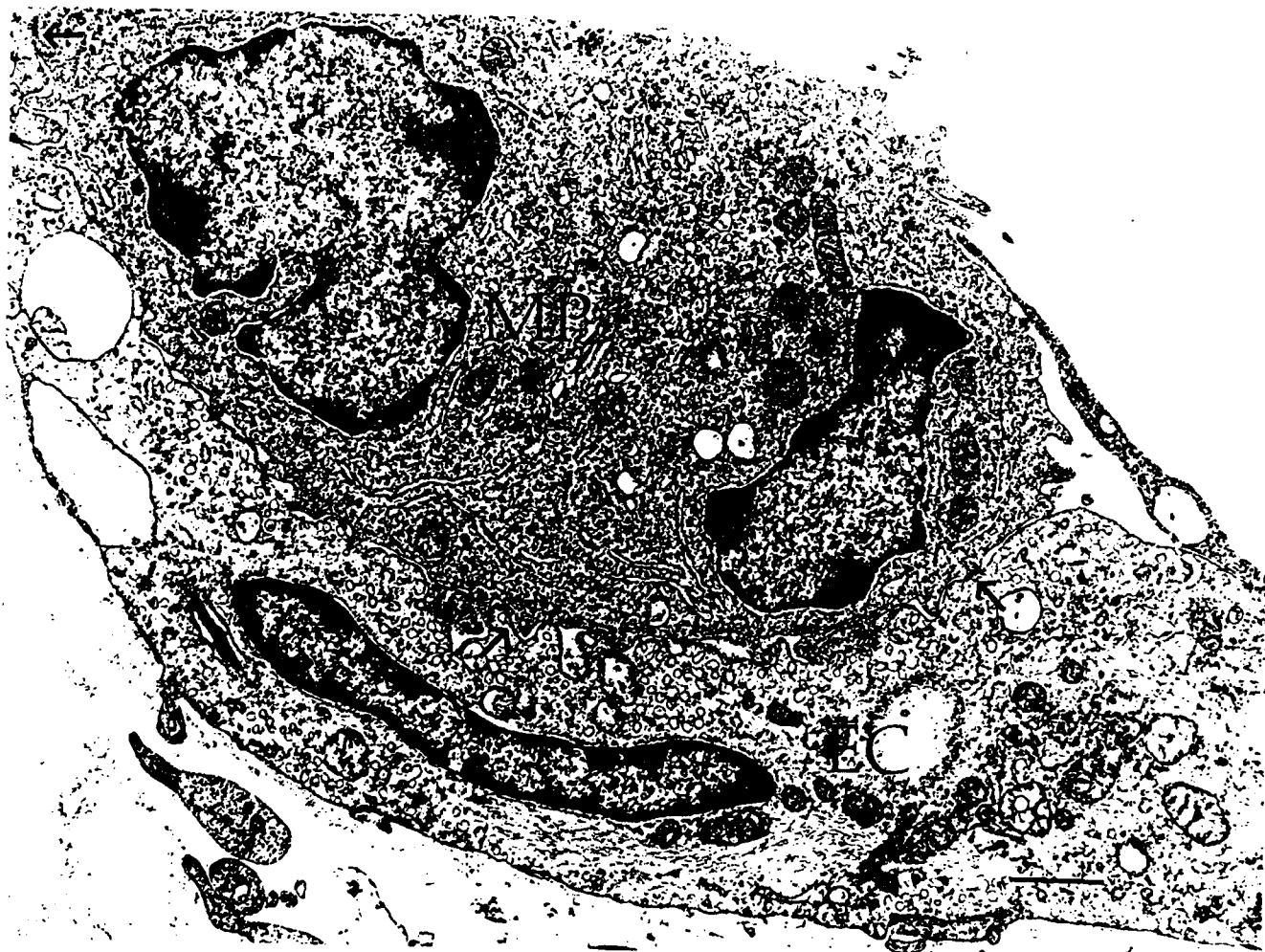


Figure 19. Transmission EM showing mononuclear phagocyte (MP) from lung microcirculation following a 4 hour incubation on the endothelial surface of a pulmonary artery explant. Endothelial cell (EC) and MP are in close apposition and demonstrate the re-establishing of three intercellular adhesion plaques (arrows). (13,800X magnification, line = 1 μ m).



Figure 20. Three separate plates of mononuclear phagocytes from porcine lung demonstrating different patterns of nonspecific esterase staining. Small arrows (A,C) point out "Rim-like" peripheral staining of cytoplasmic membrane characteristic of cells from monocyte/macrophage lineage. Cells from A, B, and C all exhibit diffuse uptake of esterase stain. Large arrow (B) shows larger cell concentrating esterase positive material in the cytoplasm along with fine esterase positive granules (1500X magnification, -naphthyl acetate).

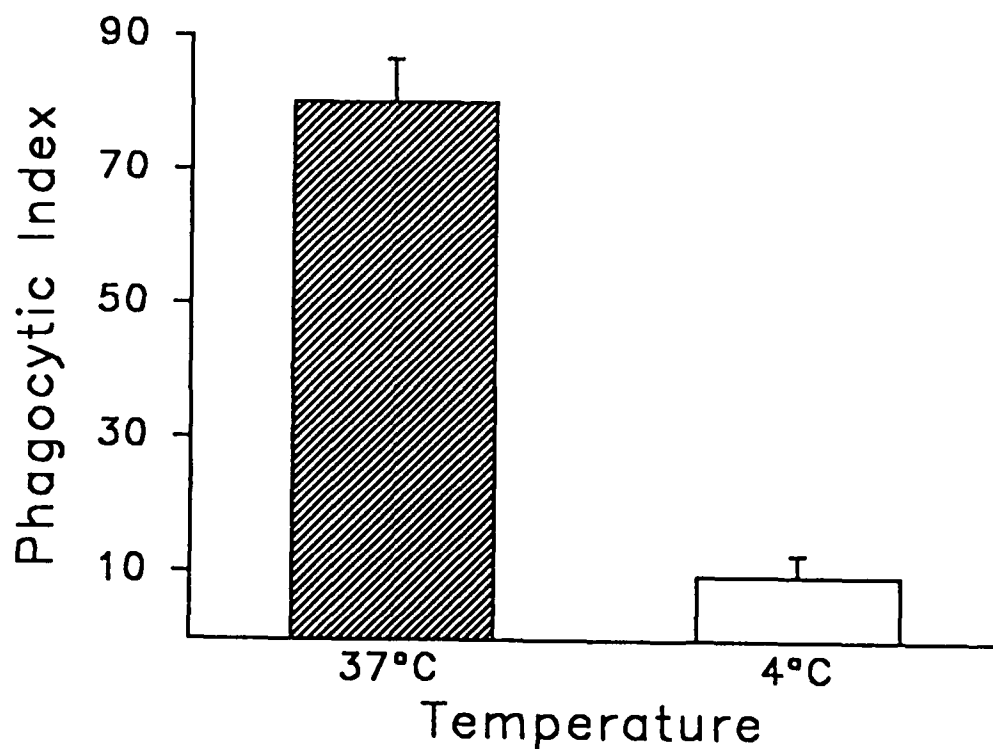


Figure 21. In-vitro phagocytosis of particulate carbon (india ink) observed in mononuclear phagocytes retrieved from microcirculation of porcine lung. At 37°C, 80 ± 6% of cells exhibited phagocytosis while cells exposed to identical particulate loads at 4°C showed only 9.5 ± 4% (P 0.01) phagocytosis.



Figure 22. Both the small (A) and large (B) constituent cells from the lung micro-circulation avidly phagocytosed particulate carbon (1500X magnification, Wright-Giemsa).

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